

Laboratory of Toxicology
Department of Forensic Medicine
University of Helsinki
Finland

DRUG ANALYSIS WITHOUT PRIMARY REFERENCE STANDARDS

Application of LC-TOFMS and LC-CLND to Biofluids and Seized Material

by
Suvi Ojanperä

*To be publicly discussed, with the permission of the Medical Faculty
of the University of Helsinki, in the auditorium of the Department of
Forensic Medicine on January 9th, 2009, at 12 noon.*

Academic dissertation

Supervised by:

Dr. Ilkka Ojanperä
Department of Forensic Medicine
University of Helsinki
Finland

Dr. Anna Pelander
Department of Forensic Medicine
University of Helsinki
Finland

Reviewed by:

Dr. Pertti Koivisto
Finnish Food Safety Authority Evira
Finland

Dr. Pirjo Lillsunde
National Public Health Institute
Finland

Opponent:

Prof. Heikki Vuorela
Division of Pharmaceutical Biology
Faculty of Pharmacy
University of Helsinki
Finland

ISBN 978-952-92-4937-4 (paperback)

ISBN 978-952-10-5181-4 (pdf)

Yliopistopaino

Helsinki 2008

CONTENTS

LIST OF ORIGINAL PUBLICATIONS 5

ABBREVIATIONS 6

ABSTRACT 7

1. INTRODUCTION 9

2. REVIEW OF THE LITERATURE 11

 2.1. Forensic and clinical drug analysis 11

 2.1.1. Areas of analysis 11

 2.1.2. Specimen considerations..... 11

 2.1.3. Sample preparation..... 12

 2.1.4. Analytical techniques 13

 2.2. Analysis without primary reference standards..... 15

 2.2.1. Availability of standards 15

 2.2.2. Identification by accurate mass measurement 16

 2.2.3. Isotopic pattern in identification..... 18

 2.2.4. Quantification..... 18

 2.3. Liquid chromatography – time-of-flight mass spectrometry (LC-TOFMS) 20

 2.4. Liquid chromatography – chemiluminescence nitrogen detection (LC-CLND) 22

3. AIMS OF THE STUDY 24

4. MATERIALS AND METHODS 25

 4.1. Reagents..... 25

 4.2. Reference standards and materials 25

 4.3. Study subjects..... 25

4.4. Methods.....	25
4.4.1. Sample preparation.....	25
4.4.2. LC-TOFMS	26
4.4.3. LC-CLND.....	27
5. RESULTS AND DISCUSSION.....	28
5.1. Urine drug screening by LC-TOFMS.....	28
5.1.1. High-throughput screening based on accurate mass.....	28
5.1.2. The effect of isotopic pattern in screening analysis	33
5.2. Analysis of street drugs	37
5.3. Quantification of drugs in blood by LC-CLND.....	40
5.3.1. Basic lipophilic drugs.....	40
5.3.2. Tramadol and metabolites.....	44
6. GENERAL DISCUSSION	47
7. CONCLUSIONS	51
8. ACKNOWLEDGEMENTS	52
9. REFERENCES.....	53

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following five articles, which are referred to by Roman numerals I-V in the text:

- I. **Pelander A, Ojanperä I, Laks S, Rasanen I, Vuori E.** Toxicological screening with formula-based metabolite identification by liquid chromatography/time-of-flight mass spectrometry. *Anal Chem* 2003;75:5710-8.
- II. **Ojanperä S, Pelander A, Pelzing M, Krebs I, Vuori E, Ojanperä I.** Isotopic pattern and accurate mass determination in urine drug screening by liquid chromatography/time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2006;20:1161-7.
- III. **Laks S, Pelander A, Vuori E, Ali-Tolppa E, Sippola E, Ojanperä I.** Analysis of street drugs in seized material without primary reference standards. *Anal Chem* 2004;76:7375-9.
- IV. **Ojanperä S, Tuominen S, Ojanperä I.** Single-calibrant quantification of drugs in plasma and whole blood by liquid chromatography – chemiluminescence nitrogen detection. *J Chromatogr B* 2007;856:239-44.
- V. **Ojanperä S, Rasanen I, Sistonen J, Pelander A, Vuori E, Ojanperä I.** Quantification of drugs in plasma without primary reference standards by liquid chromatography – chemiluminescence nitrogen detection: application to tramadol metabolite ratios. *Ther Drug Monit* 2007;29:423-8.

ABBREVIATIONS

ADC	analogue-to-digital converter
APCI	atmospheric pressure chemical ionisation
CAD	charged aerosol detection/detector
CID	collision induced dissociation
CLND	chemiluminescence nitrogen detection/detector
CNS	central nervous system
DAD	diode array detection/detector
EI	electron ionisation
EIC	extracted ion chromatogram
ELSD	evaporative light scattering detection/detector
ERETIC	electronic reference to access in vivo concentrations
ESI	electrospray ionisation
FTMS	Fourier transform ion cyclotron resonance
FWHM	full width at half maximum (resolution)
GC	gas chromatography/chromatograph
HRMS	high-resolution double-focusing magnetic-sector mass spectrometry
IDA	information dependent acquisition
IP	information point
LC	liquid chromatography/chromatograph
LLE	liquid-liquid extraction
LOD	limit of detection
log D	logarithm of distribution coefficient (octanol/water)
log P	logarithm of partition coefficient (octanol/water)
LOQ	limit of quantification
MALDI	matrix assisted laser desorption/ionisation
MRM	multiple reaction monitoring
MS	mass spectrometry/spectrometer
MS/MS	tandem mass spectrometry/spectrometer
NMR	nuclear magnetic resonance spectrometry/spectrometer
ppm	parts-per-million
PRS	primary reference standard(s)
QTOF	quadrupole time-of-flight
RRT	relative retention time
RSD	relative standard deviation
RT	retention time
SFE	supercritical-fluid extraction
SPE	solid-phase extraction
SPME	solid-phase micro-extraction
TIC	total ion chromatogram
TOF	time-of-flight
WADA	World Anti-Doping Agency

ABSTRACT

Laboratory investigation in the absence of primary reference standards is often required in forensic and clinical drug analysis for the following reasons. The standards for new drugs, metabolites, designer drugs or rare substances may not be obtainable within a reasonable period of time or their availability may also be hindered by extensive administrative requirements. Standards are usually costly and may have a limited shelf life. Finally, many compounds are not available commercially and sometimes not at all. A new approach within forensic and clinical drug analysis involves identification based on accurate mass measurement and quantification with a specific detector possessing equimolar response to nitrogen. Formula-based identification relies on the fact that the accurate mass of an ion from a chemical compound corresponds to the elemental composition of that compound. Single-calibrant quantification is feasible with a nitrogen-specific detector since approximately 90% of drugs contain nitrogen.

For qualitative analysis, reversed-phase liquid chromatography coupled with time-of-flight mass spectrometry (LC-TOFMS) was applied with an electrospray ionisation (ESI) source operated in positive ion mode. Two types of TOFMS instrumentation were in use, applying an m/z range of 100-750 (Applied Biosystems Mariner) or 50-800 (Bruker micrOTOF). For quantitative analysis, liquid chromatography coupled with chemiluminescence nitrogen detection (LC-CLND) was applied using caffeine as a single secondary standard.

A method was developed for toxicological drug screening in 1 ml urine samples by LC-TOFMS. Sample preparation consisted of enzyme hydrolysis of glucuronide conjugates and subsequent solid-phase extraction on a mixed-mode sorbent. A large target database of exact monoisotopic masses was constructed, representing the elemental formulae of reference drugs and their metabolites. Identification was based on matching the sample component's measured parameters with those in the database, including accurate mass and retention time (RT), if available. In addition, micrOTOF applied SigmaFit™, a numerical value for isotopic pattern match. Data post-processing software was developed for automated reporting of findings in an easily interpretable form. For routine screening practice, a SigmaFit tolerance of 0.03 and a mass tolerance of 10 ppm were established. Differences in ion abundance in urine extracts did not affect the accuracy of the automatically acquired SigmaFit or mass values. The limit of detection, determined for 90 compounds with Mariner, was <0.1 mg/l for 73% of the compounds studied and >1.0 mg/l for 6% of the compounds.

Seized drug samples were analysed blind by LC-TOFMS and LC-CLND, using a "dilute and shoot" approach, and results were compared to accredited reference methods. In the quantitative analysis of amphetamine, heroin and cocaine findings, the mean relative difference between the results of LC-CLND and the reference methods was 11% (range 4.2-21%), without any observable bias. The mean relative standard deviation for three parallel LC-CLND results was 6%.

Liquid-liquid extraction recoveries for basic lipophilic drugs were established by LC-CLND in blood specimens spiked with the respective reference substances. The mean recovery by butyl chloride-isopropyl alcohol extraction for plasma and for whole blood was $90 \pm 18\%$ and $84 \pm 20\%$, respectively. The validity of the generic extraction recovery-corrected single-calibrant LC-CLND was then verified with proficiency test samples. The mean accuracy was 24% and 17% for plasma and whole blood samples, respectively, and the maximum error was 31% for both specimens. All results by LC-CLND fell within the confidence range of the reference concentrations. To demonstrate the method's feasibility, metabolic ratios for the opioid drug tramadol were determined in a pharmacogenetic study setting. Four volunteers were given a single 100 mg oral dose of tramadol, and a blood sample was collected from each subject one hour later. Extraction recovery estimation was based on model compounds chosen according to their similar physicochemical characteristics (RT, pK_a , log D). The mean differences between the results of the LC-CLND and the reference method for tramadol, O-desmethyiltramadol and nortramadol were 8%, 32% and 19%, respectively.

The LC-TOFMS method allowed efficient urine drug screening with an automated target database reverse search, based on exact mass, isotopic pattern, and RT. The hit lists generated from complex data were simple and minimised the need for interpretation. Isotopic patterns, expressed as SigmaFit, revealed the true-positive findings and yielded on average 12% fewer false-positive entries than using accurate mass only. The automated acquisition of correct SigmaFit values and accurate masses were proven over a wide dynamic range, the mean mass error being only 2.5 ppm by microTOF. The combination of LC-TOFMS and LC-CLND offered a simple solution for the analysis of scheduled and designer drugs in seized material, independent of the availability of primary reference standards. In blood specimens, LC-CLND analysis, corrected for extraction recovery, produced sufficiently accurate results to be useful in a clinical context.

1. INTRODUCTION

Pharmaceuticals and drugs are an omnipresent factor of modern society, having connections to the economy, pharmacotherapy, substance abuse, drug enforcement and crime. A number of scientific and business areas are dedicated to the analysis and control of drug-like substances. Forensic toxicology is a science that generates chemical and toxicological evidence for the administration of justice mainly in the following areas: post-mortem toxicology related to cause-of-death investigations, driving under the influence of alcohol or drugs, drug-facilitated and drug-related crime, and drug testing at workplace. Forensic analysis of seized drugs is one of the duties of criminalistics and customs laboratories. Doping control in sports concerns substances on the prohibited list of the World Anti-Doping Agency (WADA). In clinical toxicology, the focus is on the diagnosis and treatment of the poisoned patient. All of the above disciplines involve similar target substances and related analytical methodologies. The techniques increasingly common in current drug analysis are based on liquid chromatography (LC), which allows the separation of a wide range of drugs, including polar and involatile compounds, and mass spectrometry (MS), which allows identification based on molecular mass and characteristic fragmentation (Van Bocxlaer 2005, Maurer 2007).

Chemical analysis measuring indirect observable properties relies largely on the use of reference standards. Analytical methods that are "ratio-based", *i.e.* require instrumental comparison with calibrants of a known quantity of the analyte, use high-purity, well-characterised primary reference standards (PRS) or species as their basis for calibration. These standards can be used either directly or through gravimetrically prepared calibration solutions (May et al. 2000). Secondary reference standards are calibrated by comparing with PRS using a high precision comparator and making appropriate corrections for non-ideal conditions of measurement. While the requirement for using PRS is obvious in quantitative analysis, these standards are the preferred reference in qualitative analysis, too.

The efficient operation of forensic and clinical laboratories is dependent on having an extensive collection of PRS on illicit and therapeutic drugs and their metabolites, as well as pesticides, household and industrial chemicals and other toxicologically relevant compounds. The number of substances required can range from a few hundred to a few thousand entries. So far, no domestic or international body has been able to provide the laboratories even with the most important substances, not to mention regularly updating them with recently launched drugs. Unfortunately, it is the task of the individual laboratories to acquire the reference substances in one of the following ways: purchasing them from commercial producers, requesting them as a gift from pharmaceutical companies or from another laboratory or scientist, or producing them by purification from seized materials. Synthesizing substances in the laboratory itself is only reasonable in isolated cases. The purchase price for a drug metabolite can be EUR 1500-2000 for 50 mg, and due to administrative requirements, the average delivery time for a reference substance is one to three months. In some cases, the material is not available at all.

Current progress in instrumental analysis promises to play a prominent role in compensating for the availability problems of reference standards. While nuclear magnetic resonance spectrometry (NMR) is too complicated for practical analytical toxicology, certain forms of MS technology offer a straightforward route to what is a fundamental property of every molecule: accurate molecular mass. Several MS techniques, including magnetic sector MS, Fourier transform ion cyclotron resonance MS (FTMS) and time-of-flight MS (TOFMS), are capable of performing accurate mass measurement (Bristow and Webb 2003). From sufficiently accurate mass, a molecular formula can be generated that, in turn, allows the assignment of candidates for substance identification. LC coupled with electrospray-ionisation orthogonal-acceleration TOF (LC-TOFMS) is a particularly promising technique in the accurate mass determination of components of com-

plex mixtures (Fang et al. 2003).

Quantitative analysis requires an LC detector capable of producing a more consistent response over a broad range of structures than the customary UV detector. Universal LC detectors include the evaporative light scattering detector (ELSD) (Yurek et al. 2002) and the corona charged aerosol detector (CAD) (McCarthy 2005). Chemiluminescence nitrogen detection (CLND), however, represents a unique approach for quantification of nitrogenous substances without PRS, because the detector possesses an equimolar response to

nitrogen (Taylor et al. 1998). This is particularly valuable in human toxicology and forensic science since approximately 90% of drugs contain nitrogen.

The present thesis investigates the use of LC-TOFMS for the identification and LC-CLND for the quantification of drugs and metabolites in biofluids and seized material, especially emphasizing analysis without PRS. The results are evaluated in the contexts of forensic and clinical toxicology and criminalistics.

2. REVIEW OF THE LITERATURE

2.1. Forensic and clinical drug analysis

2.1.1. Areas of analysis

Comprehensive screening analysis plays a key role in various fields of toxicology and forensic science (Maurer 2004). Optimal analytical performance is required in post-mortem toxicology laboratory practice related to cause-of-death investigations because of the wide scope of relevant toxicants, the multitude of specimens, sometimes severely putrefied, and the complexity of interpretation issues (Kugelberg and Jones 2007, Drummer 2007). High demands are also placed on clinical forensic toxicology, which covers the investigation of the toxicological aspects of violent crime, drug-facilitated crime, child welfare, drug use and drug trafficking (Le-Beau 2008). Identification and quantification of a wide variety of scheduled drugs in seized material is a duty of forensic science and customs laboratories (Pihlainen et al. 2003), with a strong current emphasis on drug profiling (Weyermann et al. 2008).

Controlling driving under the influence of alcohol or drugs (Walsh et al. 2004) is limited to agents that impair driving performance, yet this involves a large variety of substances. Doping control in sports focuses on the substances on the prohibited list of the WADA and is continually challenged by the emergence of new potential doping agents (Thevis et al. 2008). Drug testing at the workplace, military, prisons and schools (Lillsunde et al. 2008) is usually restricted to illicit drugs, such as amphetamines, cannabis, cocaine, heroin and phencyclidine, with supplemental therapeutic drugs that have the potential for abuse, such as benzodiazepines. Medical treatment of poisoned patients at the emergency department would also benefit from a comprehensive drug screening service (Fabbri et al. 2003), but toxicological analysis in hospitals is usually limited to rapid immunoassay techniques, due to the time requirement of approximately

two hours. Therapeutic drug monitoring refers to the quantitative analysis of patient plasma levels in order to achieve optimal drug therapy and avoid overdose (Saint-Marcoux et al. 2007).

The diversity of application fields and goals suggests that there is no single method or technique best able to deal with all forensic and clinical drug analyses (Smith et al. 2007). Nevertheless, apart from target analyses dedicated to individual key substances, a general objective has always been to develop comprehensive and universal analytical methods producing maximum information in a single run (Drummer 1999). However, the choice of method is fundamentally dependent on the specimen to be analysed and on the sample preparation involved.

2.1.2. Specimen considerations

The reliability and relevance of analytical toxicology results is determined by the nature and integrity of the specimens submitted for analysis (Flanagan et al. 2005). Screening analysis is often performed in urine, because the time-window of detection is longer in urine than in blood. A sole urine sample may suffice in some areas of analytical toxicology, such as in drug testing and clinical toxicology. Blood concentration reflects best the acute action of a substance, and consequently, extensive compilations of therapeutic, toxic and lethal drug concentrations have been published for blood, plasma and serum (Schulz and Schmoldt 2003). Blood sampling is necessary if the level of intoxication is to be studied.

Lipophilic compounds exist in urine mainly as metabolites, which are more hydrophilic than the parent compound and thus more readily excreted via the kidneys. Phase I metabolism commonly involves oxidation, reduction and hydrolysis, while phase II metabolism involves conjugation reactions, especially glucuronidation and sulphation. For hydrophilic compounds that are not metabolised, the concentrations are usually high in urine. A cut-off concentration differenti-

ating a positive from a negative finding should be specified particularly for drugs-of-abuse in drug testing programs (Lillsunde et al. 2008). Exact urine drug concentrations usually have a very limited interpretative value, except at the vicinity of the cut-off value. The cut-off is an administrative value not necessarily equal to the analytical limit of detection (LOD) or limit of quantification (LOQ). The cut-off value established will subsequently determine the time-window of detection after drug exposure (Reiter et al. 2001, Verstraete 2004).

Although blood and urine are the most important specimens for toxicological drug analysis, other materials, such as hair, saliva, sweat and meconium are also commonly used (Dolan et al. 2004). In post-mortem toxicology, the vitreous humor is a good alternative to urine, which is not always available. Saliva is now extensively used in the control of driving under the influence of drugs due to the non-invasive sampling procedure. Hair is another popular material, especially in clinical forensic toxicology. Drugs incorporated in the hair can reveal past drug use even after several months.

2.1.3. Sample preparation

The standard handbook on analytical toxicology by Baselt (Baselt 2004) covers approximately 600 relevant organic toxicants, of which about 80% contain nitrogen. Major drugs of abuse that do not contain nitrogen are few; they include gamma-hydroxybutyrate and cannabinoids. Sample preparation for drug screening usually involves division of the analytes into basic and acidic fractions. The basic fraction contains the most important, toxicologically relevant classes of drugs and pharmaceuticals, especially substances that act on the central nervous system (CNS), such as antidepressants, antipsychotics, benzodiazepines, opioids and stimulants. Important cardiovascular drugs are also mostly basic in nature, including adrenergic beta-blocking drugs, antiarrhythmics and calcium channel blockers. In scientific drug discovery, general structural descriptors have been associated with drug-like-

ness (Vistoli et al. 2008). Summarising from several sources, an average range of properties for a target compound of drug analysis can be established: it is a nitrogenous, basic compound with a molecular mass ranging 150-500, lipophilicity as log P ranging 1-5, and water solubility of 50 mg/l at pH 6.5 (Moffat et al. 2003, Baselt 2004, Vistoli et al. 2008).

Direct determination of drugs by chromatographic analysis is usually impossible due to the complexity of the sample, which often demands a sample preparation step that is time-consuming, tedious, and frequently neglected. Compounds possessing phenolic or alcoholic hydroxyl groups exist in urine as conjugates, making a hydrolysis step necessary prior to extraction. These compounds include many toxicologically important drugs, such as opiates, benzodiazepines and cannabis. Enzyme hydrolysis is commonly used, because acid or base hydrolysis, though rapid, requires harsher conditions and also produces unwanted reactions. Several sample preparation techniques are used for drug analysis, including liquid-liquid extraction (LLE), supercritical-fluid extraction (SFE), solid-phase extraction (SPE), solid-phase micro-extraction (SPME), liquid-phase micro-extraction, and use of restricted access materials (Wille and Lambert 2007). Screening for hundreds of basic drugs necessitates a general, universally applicable extraction method, instead of a series of selective target methods adjusted for individual compounds. For quantitative analysis in blood, conventional LLE obviously still provides the most robust performance, while simple protein precipitation prior to LC analysis has also been found useful (Pragst et al. 2004, Flanagan et al. 2006). SPE is more amenable to automation, but it has a higher number of variables to be optimised, which may result in poor quantitative precision. SPE is particularly well suited for urine, in which cells and proteins are not a problem. For applying reversed phase or mixed mode sorbents that combine reversed phase and ion exchange, SPE is a good choice in comprehensive urine drug analysis (Decaestecker et al. 2003). SPME has been found to be effective in various target analyses (Pragst 2007), and SFE is particularly suitable in cases involving ex-

traction from hair (Brewer et al. 2001).

Direct on-line injection methods offer the advantage of reducing sample preparation steps and enabling effective pre-concentration and clean-up of biological fluids. These methods involve restricted-access materials and other automated on-line SPE procedures. Emerging automated extraction-phase technologies include molecularly imprinted polymers, in-tube solid-phase microextraction, and microextraction in a packed syringe for more selective extraction (Mullett 2007). Seized samples often contain a high proportion of the active substance and consequently do not necessarily require concentrating sample work-up. However, LLE procedures are useful in separating impurities and adulterants from active substances (King et al. 1994). The success of a particular extraction method very much depends on the subsequent analysis method, and this is especially true in bioanalysis, where endogenous background and matrix effects are a major concern.

2.1.4. Analytical techniques

In qualitative drug screening, techniques based on gas chromatography-mass spectrometry (GC-MS) have long provided the best and most cost-effective performance. This is largely due to the reproducible nature of the electron ionisation (EI) MS spectrum, allowing the construction of spectral libraries in-house or on an interlaboratory basis. Several extensive EI-GC-MS libraries, containing thousands or hundreds of thousands of spectra, are commercially available to facilitate broad-scale screening for organic low-molecular-weight compounds, including drugs, designer drugs, poisons and pesticides. GC-MS has been referred to as “the gold standard” of toxicological analysis. This honour is mainly due to the strength of GC-MS in confirming urine immunoassay results for drugs of abuse.

LC combined with diode array UV detection (LC-DAD) is a standard technique in therapeutic drug monitoring, and it has also been found to be useful in comprehensive drug screening. In the development of UV spectral libraries for

identification purposes, great attention has to be paid to the accuracy and precision of retention parameters. An LC identification system based on a 1-nitroalkane retention index standard scale was described for 383 toxicologically relevant compounds using a reversed-phase column (Bogusz and Erkens 1994). Another index standard series involved drug substances as secondary standards (Elliott and Hale 1998). The identification power of UV spectra and retention index, applied separately, was low, but increased substantially when the two parameters were used in combination (Maier and Bogusz 1995). A very comprehensive LC-DAD method, relying on relative retention time (RRT), included 2682 substances (Herzler et al. 2003). Although the use of RRT alone produced unsatisfactory identification results, 1619 substances (60.4%) were unambiguously identified by their UV spectra only. This rate was increased to 84.2% by the combination of spectrum and RRT. The authors concluded that LC-DAD is one of the most reliable methods for substance identification in toxicological analysis.

During the past two decades, LC-MS has gained increasing success in analytical toxicology. Most of the methods developed at the beginning were target analyses for a limited number of analytes, typically for a drug and its main metabolites (Hoja et al. 1997). Comprehensive screening methods started to appear in the scientific literature only ten years ago for pesticides (Slobodnik et al. 1996) and drugs (Marquet et al. 2000). Several types of LC-MS techniques have been applied to drug screening, notably based on single or triple quadrupole, ion-trap and time-of-flight (TOF) mass analysers (Maurer 2004).

Utilisation of low-resolution MS techniques requires that either the target analytes' spectra have been recorded in the spectral libraries used or the analyst possesses the respective PRS for direct comparison. MS fragmentation patterns in GC-MS (Peters et al. 2003) and ion-trap multiple LC-MS (Kölliker and Oehme 2004) have been described to aid the structural elucidation of amphetamines, but this approach is overly complicated for everyday forensic casework. Newly developed software can predict mass fragmen-

tation from chemical structure according to different ionisation techniques and fragmentation rules (Stranz et al. 2008), but these programs are not routinely used.

Comprehensive library-based screening methods have been developed by using single quadrupole LC-MS with collision-induced dissociation (CID) taking place in-source (Weinmann et al. 1999, Rittner et al. 2001, Saint-Marcoux et al. 2003). Typically, spectra obtained at positive and negative polarity were created simultaneously at a high and low orifice voltage, showing extensive and weak fragmentation, respectively, and the spectra were summed at both polarities to produce informative spectral libraries. As single MS methods suffer from lack of specificity in distinguishing co-eluting substances, much emphasis should be put on chromatographic resolution. In addition, there may be difficulty in harmonising the CID conditions for producing reproducible spectra (Bogusz et al. 1999).

More reliable identification can be obtained by LC coupled to tandem MS (MS/MS) with triple quadrupole (Gergov et al. 2000, Weinmann et al. 2000), hybrid quadrupole/ion-trap (Marquet et al. 2003), or hybrid quadrupole/TOFMS (QTOF-MS) technology (Decaestecker et al. 2004). This is due to the fact that the LC-MS/MS product ion spectra generated by these techniques are less dependent on sample composition and experimental settings. However, the product ion scan approach requires the choice of a limited number of ions to be monitored in the first quadrupole, which makes a separate survey scan necessary to select the ions (Gergov et al. 2001b). Information dependent acquisition (IDA) is an interesting approach in comprehensive screening analysis by LC-MS/MS. It consists of three steps – acquisition of survey data, selection of the parent ions of interest by abundance, and monitoring the product ion spectra – all in a single run. IDA was first described using a QTOF-MS instrument, without actually utilizing accurate mass measurement, however, (Decaestecker et al. 2000, Decaestecker et al. 2004), and later applied by others using quadrupole/ion-trap instruments (Marquet et al. 2003, Mueller et al. 2005). A major advantage of IDA is high specificity and selectivity of analy-

sis, but problems may arise with variable background noise, resulting in difficulties in setting the abundance threshold for acquisition.

The reproducibility of LC-MS/MS spectra between various brands of instruments has often been questioned. Several methods for standardising libraries of spectra have been investigated between various brands or techniques, including triple quadrupole instruments (Gergov et al. 2004), hybrid quadrupole/ion-trap instruments (Mueller et al. 2005) and many types of LC-MS/MS instruments (Bristow et al. 2004). In a recent interlaboratory study, the spectra of 48 compounds were recorded on eleven mass spectrometers, including six ion-traps, two triple quadrupoles, a hybrid triple quadrupole, and two QTOF-MS instruments (Hopley et al. 2008). The reproducibility of the product ion spectra was increased when considering the tandem-in-time instruments and the tandem-in-space instruments as two separate groups. A more limited screening library was proposed for LC-MS/MS identification using instruments of the same type from different manufacturers (Hopley et al. 2008). Both MS/MS product ion spectra and in-source-CID-MS spectra have been made commercially available, but thus far they have found little use compared to the success of EI-GC-MS libraries.

Another mode of analysis within LC-MS/MS that has been applied to screening analysis is multiple reaction monitoring (MRM). It is a more sensitive mode of operation and amenable to quantification, but there is also a technical limit to the number of target compounds that can be monitored by MRM-type experiments, which hinders the utility of this approach in comprehensive screening. A qualitative screening method for up to 238 drugs has been published using MRM (Gergov et al. 2003), but usually MRM methods comprise much fewer compounds.

The advantages and disadvantages of LC-MS screening methods are much related to the commonly used ionisation techniques, atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) (Fenn et al. 1990, Bruins 1991). Both are soft ionisation techniques that do not produce much fragmentation. Vol-

atile buffers are required for chromatography, which limits the optimisation of separation by the modifications of mobile phase. Neither technique is universal for all types of compounds, although ESI possesses a broader applicability in drug analysis due to its suitability for polar compounds. Unfortunately, there is always the risk of detrimental ion suppression, particularly with ESI (Dams et al. 2003), and consequent false negative findings in comprehensive screening analysis, where matrix effects cannot be controlled for the numerous target compounds included in the screen.

Simultaneous screening and quantification is viable mainly when using techniques allow quantitative calibrations to be performed only infrequently, *i.e.* on a weekly or monthly basis, such as by GC with nitrogen-selective detection (Rasanen et al. 2003) or by LC with diode-array UV detection (Pragst et al. 2004). A limit of 100-200 compounds is the maximum in such methods in order to maintain both qualitative and quantitative precision in practical work. Evidently, precision of the chromatographic retention parameter plays a more prominent role in GC- and LC-based methods than in the MS-based methods discussed above. For analytic techniques that need quantitative calibration in each sequence of runs, especially GC-MS and LC-MS, the methods used have typically consisted of only 10-20 compounds. However, a quantitative method has been developed for up to 100 pesticides in food with triple quadrupole LC-MS/MS, using a simplified calibration scheme (Ferrer et al. 2007).

2.2. Analysis without primary reference standards

2.2.1. Availability of standards

Analysis of drugs is hindered by the lack of readily available PRS, which are required when using techniques in which identification and quantification are based on comparison of chromatographic retention, spectra and detector response between the analyte and the standard. While extensive collections of spectral and chromatographic data are available for common drugs

(Herzler et al. 2003, Moffat et al. 2003), problems arise when rare and new substances appear on the continually changing drug scene. The term designer drug originated in connection with the epidemic of unscheduled fentanyl analogs in California during the 1980s (Henderson 1988). Stricter analog legislation in the US since 1986 probably prompted the relocation of designer drug production, largely to Europe. The following chemical classes are prominent among the drugs encountered: amphetamines (Peters et al. 2003), fentanyls (Ohta et al. 1999), piperazines (de Boer et al. 2001, Peters et al. 2003), pyrrolidinopropiophenones (Springer et al. 2003) and tryptamines (Shulgin 2003).

Another area of research that suffers from the lack of PRS is metabolite analysis. With many drugs, such as heroin and cocaine, the metabolites are the main proof of illicit drug intake (Jones et al. 2008). In blood, the ratio of parent drug to metabolite is instrumental in estimating the time of administration, in differentiating between acute vs. chronic exposure and in studying pharmacogenetic aspects. Metabolite findings in urine are also helpful in confirming drug screening results. Finding a metabolite in hair can verify that the corresponding drug has been ingested, if external contamination had been suspected instead.

PRS for certain drugs and metabolites are available from commercial, governmental and international sources. However, their delivery time is lengthy, normally ranging from several weeks to several months. Moreover, the cost of standards is further increased by the extensive administrative requirements imposed on the importation or exportation of controlled substances. In many cases, PRS are not commercially available. The considerable effort required for synthesising PRS in-house is justified in important research-oriented projects. This is exemplified with the synthesis of amphetamine impurities for the development of a harmonised method for profiling amphetamines (Aalberg et al. 2005) and with the synthesis of steroid metabolites for predicting the metabolic patterns of new derivatives of anabolic androgenic steroids for doping control purposes (Kuuranen et al. 2008).

Apart from designer drugs and metabolites, it may even be problematic to acquire a PRS for a newly launched prescription drug because of the ever-increasing cost of drug development and the subsequent reluctance of the manufacturer to release the standard. Forensic and toxicological investigations are by nature prone to produce undesirable results by revealing trends of poisonings that may threaten the market share of the drug. The history of forensic toxicology recognises a number of prescription drugs that have been found to be associated with abuse or an excess of fatal poisonings, such as dextropropoxyphene (Hudson et al. 1977), barbiturates (Stead and Moffat 1983), tricyclic antidepressants (Henry 1989), buprenorphine (Tracqui et al. 1998), venlafaxine (Koski et al. 2005) and tramadol (Tjäderborn et al. 2007).

2.2.2. Identification by accurate mass measurement

In the 1950s, Beynon explained for the first time that if the mass of an ion from a chemical compound is determined with sufficient accuracy, the elemental composition of that compound

could be deduced (Beynon 1954). Mass accuracy is the difference between the theoretical value of the mass of an ion and the mass measured using a mass spectrometer. The resolving power of a mass spectrometer is defined as the capacity to separate ions of adjacent m/z , and resolution is the measure of the separation of the two mass spectral peaks. Resolution is usually defined in one of two ways, depending on the mass spectrometer being used (Figure 1). The 10% valley (intensity) definition used with magnetic sector instruments states that two peaks of equal intensity are considered to be resolved when they are separated by a valley, which is 10% of the height of each peak. The definition used with quadrupole, Fourier transform ion cyclotron resonance, ion-trap and time-of-flight mass spectrometers is based on a peak width measured at 50% peak height (full width at half maximum or FWHM), producing a value approximately double that calculated using the 10% valley definition (Bristow 2006). The better the accuracy, the less the ambiguity in molecular formula determination. With increasing m/z , the number of formulae fitting a measured molecular mass will increase until it becomes impossible to obtain an unambiguous result (Webb et al. 2004) (Figure 2).

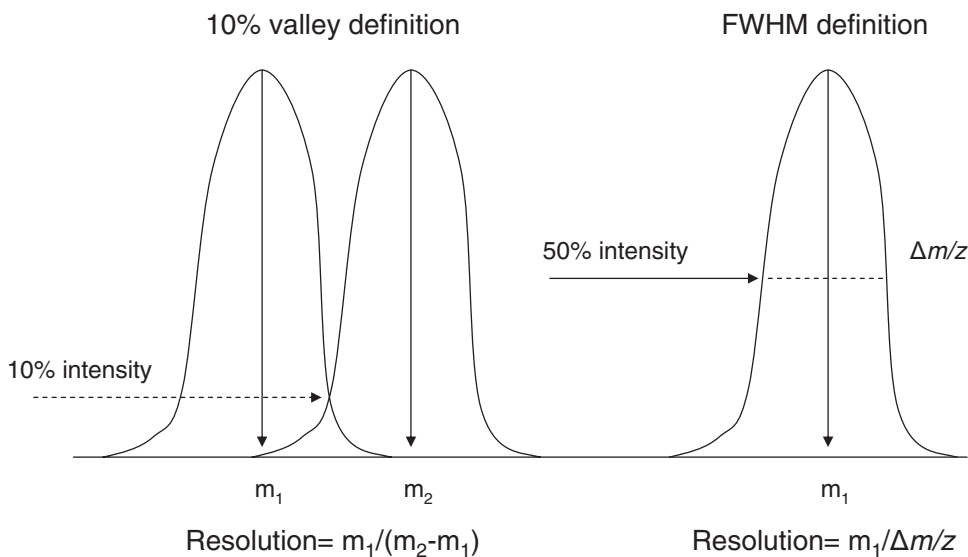


Figure 1. Two ways of defining the mass resolution: the 10% valley definition and the full width at half maximum (FWHM) definition.

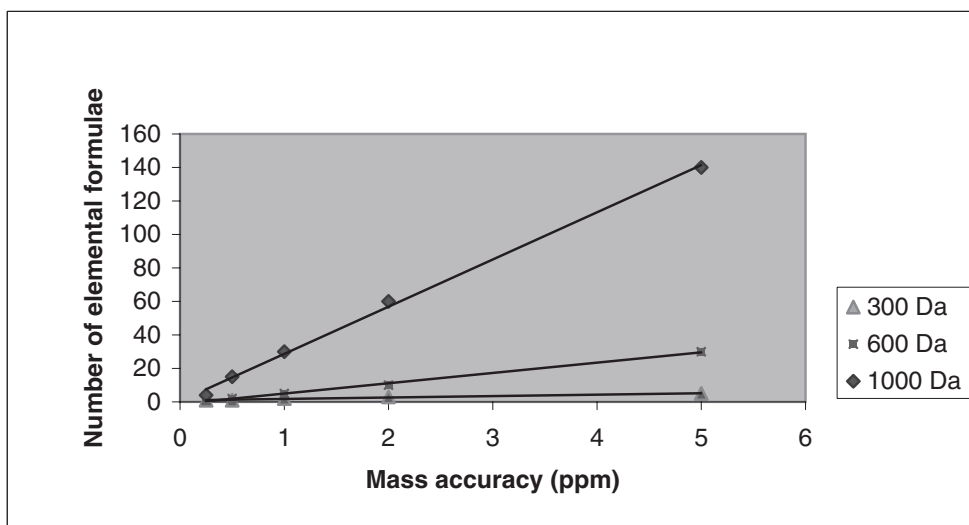


Figure 2. Relation of mass accuracy and the number of possible elemental formulae at three different molecular weights.

For many years since the discovery of the accurate mass concept, measurements were only carried out using magnetic-sector MS. The availability of such measurements was limited due to the cost and complexity of the instrumentation and the need for considerable expertise to acquire and interpret the spectra (Bristow 2006). Already in the 1970s, accurate mass was utilised for monitoring specific compounds in environmental and biological samples with packed-column (Kimble et al. 1974, Ehrenthal et al. 1977) and capillary column GC coupled to high-resolution double-focusing magnetic-sector MS (GC-HRMS) (Burlingame 1977, Lewis et al. 1979). Still today, the GC-HRMS technique is in use in certain demanding applications, such as in investigating human exposure to dioxins and PCB compounds (Tuomisto et al. 2006). Using the peak matching mode, an accuracy of <1 ppm can be achieved, but the reference mass should be as close as possible to the analyte mass, thus excluding broad-spectrum mass screening (Bristow and Webb 2003).

Single-quadrupole and triple-quadrupole MS are inherently low-resolution instruments. However, accurate mass measurements (<5 ppm) can be obtained after careful calibration in a narrow m/z range and when no unresolved interferences are present at the masses of interest (Tyler et al. 1996). This was emphasised in an interlabora-

tory comparison where both of the participating laboratories using triple-quadrupole instruments reported a mean measurement accuracy of <2 ppm (Bristow and Webb 2003).

Recently, a novel hybrid mass spectrometer was described. It couples a linear ion-trap mass spectrometer to an orbitrap mass analyser via a radio-frequency-only trapping quadrupole with a curved axis. The instrument provided high resolution and high-accuracy mass measurements, within 2 ppm using internal standards and within 5 ppm with external calibration (Makarov et al. 2006). However, the total MS cycle time was highly dependent on mass resolution; *i.e.*, the higher the mass resolution, the higher the cycle time. For example, a mass resolution of 100,000 (FWHM) resulted in a cycle time of about 2 s. This excludes the use of ultra high-performance LC separations, because there will not be enough data points over a chromatographic peak (Hogenboom et al. 2008).

The ultimate mass measurement technique is FTMS, which provides very high mass accuracy (<1 ppm) and mass resolving power (Amster 1996, Marshall et al. 1998, Bristow and Webb 2003) as well as high sensitivity in the attomole and even in the zeptomole range (Belov et al. 2000). The high mass accuracy of FTMS has been used for several years for the accurate mass tag

strategy in proteomics to detect proteins using exact masses of protein tryptic digests (Smith et al. 2002). Results obtained in toxicology have shown that very accurate masses can dramatically improve confidence in identifying compounds using a database search (Ojanperä et al. 2005).

Accurate mass determination with moderately good resolution became feasible on a routine basis with the development of orthogonal acceleration TOFMS analysers (Dawson and Guilhaus 1989), and modern instruments can readily be combined with LC using an ESI ion source (Mirgorodskaya et al. 1994). Affordable benchtop LC-TOFMS instruments have found widespread use in the analysis of small molecules, such as in drug bioanalysis (Zhang et al. 2000a) and in high-throughput screening for combinatorial chemistry libraries (Fang et al. 2003). Emerging TOFMS technology, resulting in continual improvements in mass accuracy, resolution and other analytically important features, has greatly diminished the development of magnetic-sector MS (Guilhaus et al. 2000). A key concept of the present thesis involves a LC-TOFMS urine drug screening method that essentially relies on accurate mass measurement combined with an automated formula-based target database search (Gergov et al. 2001a). A detailed discussion of LC-TOFMS is included in Section 2.4.

2.2.3. Isotopic pattern in identification

In addition to accurate mass, isotopic pattern is a property that can be utilised in substance identification. Many elements have typical isotope patterns, allowing the number of possible elemental formulae to be reduced. The following elements have distinctive patterns: chlorine ($^{35}\text{Cl}:^{37}\text{Cl} \sim 3:1$), bromine ($^{79}\text{Br}:^{81}\text{Br} \sim 1:1$) and sulphur ($^{32}\text{S}:^{33}\text{S}:^{34}\text{S} \sim 100:1:4$). The carbon isotope ratio ($^{12}\text{C}:^{13}\text{C} \sim 100:1.1$) can be used to calculate the number of carbon atoms in the molecule. An ion containing 10 carbon atoms will have a ^{13}C isotope peak with an abundance of 11% of the ^{12}C peak and an ion containing 50 carbon atoms will have a ^{13}C isotope peak with

an abundance of 55% of the ^{12}C peak (Bristow 2006). The calculation of abundance patterns resulting from the combination of more than one polyisotopic element becomes more complex. Matching of the theoretical calculated isotopic pattern against the measured pattern plays a key step in revealing the correct elemental composition from a mass spectrum (Rockwood and van Orden 1996). The idea was discovered years ago and has proven useful for structure elucidation using low-resolution mass spectra (Kavanagh 1980, Tenhosaari 1988, Palmer and Enke 1989). However, prior to the present thesis, the use of isotopic patterns as a numerical identification parameter has not been investigated in the context of drug screening.

2.2.4. Quantification

While qualitative analysis without PRS can lean on accurate mass measurement, spectral and chromatographic libraries and ultimately on NMR spectrometry, quantification has been very much dependent on PRS. Thus, maintaining an up-to-date collection of hundreds of standards is a tedious and expensive duty of forensic and clinical laboratories. Determination of blood drug concentrations is a necessity for the evaluation of whether a person has been under the influence of a substance. In forensic science, quantitative information on the purity of illegal drugs is used for various purposes, including valuation for sentencing, profiling and sample comparison, in addition to use in studies on the economics of the illicit market (King 1997).

There are few analytical techniques capable of accurate quantification without PRS. An important approach is quantification by proton counting with NMR. The electronic reference to access in vivo concentrations (ERETIC) method in NMR was introduced as a way of determining absolute concentrations (Barantin et al. 1997). An artificial radio frequency reference ERETIC signal produced with rigorously controlled parameters is added to the observed NMR sample spectrum. This eliminates handling and contamination issues associated with internal standards,

allowing quantification of protons with respect to the ERETIC signal. High accuracy and precision was obtained with the ERETIC method in NMR, referred to as a “gold standard” of quantification (Lane et al. 2005, Lane et al. 2006). However, this technique is beyond the scope of most forensic and clinical laboratories.

In GC, the concept of effective carbon number is a model by which the flame ionisation response of compounds can be predicted, and it has been utilised for cannabinoids (Poortman-van der Meer and Huizer 1999) and amphetamine-type compounds (Huizer et al. 2001). Using individually selected secondary standards for each type of stimulant, the error of prediction was generally less than 5% (Huizer et al. 2001).

In combinatorial chemistry related to drug discovery, the characterisation of synthesis products, without possessing PRS, has become feasible via utilisation of special detectors, such as the evaporative light scattering detector (ELSD) (Fang et al. 2000) or the chemiluminescence nitrogen detector (CLND) (Taylor et al. 1998, Yurek et al. 2002, Yan et al. 2003). ELSD (Ford and Kennard 1966) is an excellent quantitative detector when calibrated in an analyte-specific manner. LC-ELSD detects all compounds that are less volatile than the mobile phase, and it is commonly used for compounds without a UV chromophore, such as carbohydrates, lipids and polymers. ELSD can therefore offer advantages over conventional UV or refractive index detection, particularly for gradient separations. The general detection mechanism of ELSD involves a three-stage process: nebulisation, evaporation, and detection. The aerosol formed enters a heated evaporator tube, where the mobile phase is evaporated, leaving a dry particle plume. The dry particles coming out from the tube are irradiated by a light source, and the scattered light from the particles is detected. The quantity of light scattered by the particles is dependent on the concentration of the analyte, and the ELSD response is related to the absolute quantity of the compound independently of its optical properties (Zhang et al. 2008). Careful choice of standards could limit errors to 10–20% for limited sample sets (Kibbey 1996). However, larger errors occurred in cases

where standards were less representative (Fang et al. 2000), and reduced ELSD response was obtained from low-molecular-weight compounds (Hsu et al. 1999, Fang et al. 2001). ELSD was found to normally bias toward the underestimation of chromatographically resolved impurities, resulting in an overestimation of analyte purity (Lane et al. 2005)

Another universal detector, the corona charged aerosol detector (CAD), was first developed in 2004 (Paschla 2005), but little critical scientific literature is available on this instrument yet (Gorecki et al. 2006, Brunelli et al. 2007). In LC coupled to corona CAD, the mobile phase is nebulised with nitrogen and the droplets are dried, producing analyte particles. A stream of nitrogen becomes positively charged as it passes a high-voltage platinum corona wire, and this charge is transferred to the opposing stream of analyte particles. The charge is then measured at a collector, generating a signal with an intensity that is proportional to the quantity of analyte. Corona CAD is more sensitive than ELSD and has a wider dynamic range; it also allows the use of a wider variety of mobile phases and buffers. However, as with ELSD, the response depends on the composition of the mobile phase, with higher responses observed at higher organic contents (Zhang et al. 2008). In LC applications, a technique called mobile-phase compensation can be used to solve this problem (Gorecki et al. 2006). A separate pump is required to compensate the organic content in the mobile phase by delivering exactly an inversed gradient prior to the detection. As long as the compounds are non-volatile, the response factors obtained on corona CAD are uniform independently of their nature, which opens the door to quantification of unidentified species or single-compound calibration.

CLND is a detector that has already achieved an established position in the science of drug discovery due to its equimolar response to nitrogen-containing compounds, allowing the detector to be calibrated by a single secondary standard (Yan 1999). The detector is thus a promising alternative for managing with the challenges of forensic and clinical drug analysis. A detailed discussion of LC-CLND is included in Section 2.5.

2.3. Liquid chromatography – time-of-flight mass spectrometry (LC-TOFMS)

TOFMS has been commercially available since the late 1950s, following the publication of the design later commercialised by the Bendix Corporation (Wiley and McClaren 1955). The TOFMS is an attractive instrument due to its potentially unlimited m/z range and high-speed acquisition capabilities. However, there were several physical and technical limitations to the early TOFMS instruments that limited their resolving power (FWHM 300) and mass accuracy (Guilhaus et al. 1997). The initial spatial, temporal, and velocity distributions of ion populations can broaden the distribution of ion arrival times, hence greatly reducing resolving power. This has an effect on mass accuracy as there is a high probability that interfering ions will not be resolved. Three major technological developments have resulted in TOFMS becoming capable of accurate mass measurement: the reflectron, delayed extraction, and orthogonal acceleration (Bristow 2006). The rapid growth of

applications involving TOFMS is due to the emergence of matrix-assisted laser desorption/ionisation (MALDI) and the rediscovery of the orthogonal acceleration concept (Guilhaus et al. 2000).

Off-axis or orthogonal acceleration TOFMS of ions from continuous ion beams has been known since the 1960s. It was only in the late 1980s and early 1990s that the current range of TOFMS instruments with greatly improved resolving power and mass accuracy was developed (Dawson and Guilhaus 1989, Guilhaus et al. 1997). The key features enabling accurate mass measurement include high efficiency in gating ions from an external continuous source (e.g. ESI, APCI), simultaneous correction of velocity and spatial dispersion, and increased mass resolving power (Bristow 2006). The digital electronics revolution has supported TOFMS more than MS technologies more heavily reliant on analog signal processing (Guilhaus et al. 2000). In this dissertation, TOFMS refers to orthogonal acceleration technology unless otherwise stated. A schematic presentation of orthogonal acceleration TOFMS is shown in Figure 3.

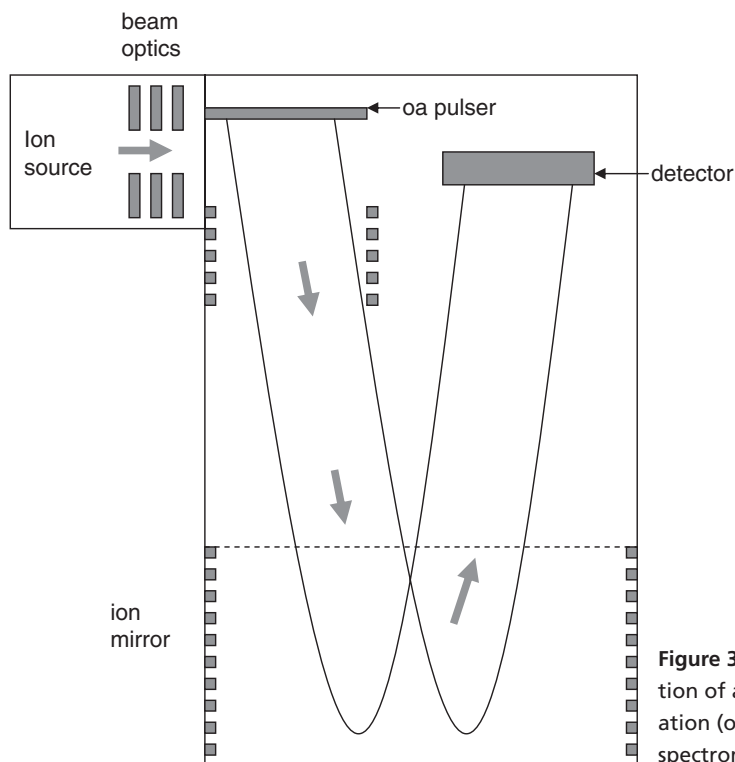


Figure 3. A schematic presentation of an orthogonal acceleration (oa) time-of-flight mass spectrometer (TOFMS).

LC-TOFMS enables accurate mass determination of components of complex mixtures to be performed in a routine manner (Chernushevich et al. 1997, Guilhaus et al. 1997, Eckers et al. 2000). In the field of drug discovery, the development of combinatorial chemistry created a need for rapid characterisation of the complex mixtures that are generated by synthesis (Yurek et al. 2002). Two MS techniques, LC-FTMS (Fang et al. 1998) and LC-TOFMS (Fang et al. 2002), made feasible accurate mass measurement of even thermally unstable and higher-molecular-mass compounds. Currently, LC-TOFMS is the most cost-effective technique for performing accurate mass analysis of small molecules on a routine basis (Balogh 2004). In addition to high mass accuracy (<5 ppm), the benefits of TOFMS include good mass resolution (FWHM 10,000), wide mass range, and fast mass spectral acquisition speed with high full-scan sensitivity — all attributes being superior to those obtained with a scanned quadrupole. Today, the mass accuracy of TOFMS is comparable to that of much more expensive accurate-mass instruments (Stroh et al. 2007), and the mass resolution can exceed 60,000 FWHM.

LC-TOFMS-based accurate-mass methods have already found extensive use in many facets of analytical research, for example, in the structure elucidation of metabolites (Zhang 2000b, Nassar et al. 2003, Leclercq et al. 2005), pesticides (Maizels and Budde 2001), steroids (Nielen et al. 2001, Nielen et al. 2007), and unknown compounds in environmental water (Ibanez et al. 2005). Notably, comprehensive screening analysis had not been realised until the studies of this thesis, mainly due to limitations in data acquisition and processing capabilities. Recently, however, an analogous multi-residue monitoring approach involving in-source CID has been published for the analysis of pesticides and their degradation products in food and water samples (Ferrer and Thurman 2007).

For drug screening purposes, it is very important to be able to measure the accurate mass of the sample components in an automated manner, without successive dilutions of the sample or tedious optimisations for each ion depending on

its abundance. New generation TOFMS instruments use detection systems based on analog-to-digital converters (ADC). An advantage of this technology is a much increased ion-abundance dynamic range for accurate mass measurement. An alternative approach that still employs time-to-digital technology is to defocus the ion beam, resulting in reduced abundance at the detector. Therefore, higher analyte concentrations and hence higher ion abundance can be measured at the optimum ion abundance for the detector (Bristow et al. 2008).

The development and commercialisation of hybrid QTOF-MS used a similar approach to orthogonal acceleration TOFMS (Morris et al. 1996, Chernushevich et al. 2001). In a recent evaluation of a modern QTOF-MS instrument applying ADC technology, mass measurement accuracy remained stable, within ± 0.0015 *m/z* units, over approximately 3–4 orders of magnitude of ion abundance. In MS/MS experiments, similar mass accuracy to single MS was obtained for product ions using only one calibration procedure. However, it was slightly reduced at low ion abundance (Bristow et al. 2008). These findings suggest that QTOF-MS is an equally feasible instrumentation for drug screening based on accurate mass measurement.

Current TOFMS instrumentation has been equipped with isotopic pattern match algorithms as a part of the molecular formula generation capabilities, providing an exact numerical comparison of theoretical and measured isotopic patterns as an additional identification tool for accurate mass determination. In a preliminary study, this function was found to facilitate urine drug screening in distinguishing between compounds with adjacent molecular masses (Laks et al. 2004). Later, it was shown that isotopic pattern match can also be successfully used with MS/MS experiments using a QTOF-MS instrument (Bristow et al. 2008).

2.4. Liquid chromatography – chemiluminescence nitrogen detection (LC-CLND)

Chemiluminescent reactions have a history of over one hundred years, and analytical applications of chemiluminescence began to be used in the 1970s. The chemiluminescent reactions between ozone and various molecules have been exploited for detection of olefins, ozone, nitric oxide, other nitrogen oxides, organosulphur compounds, and a variety of other organic compounds. $\text{NO} + \text{O}_3$ chemiluminescence forms the basis of a large number of these applications. At first, many of the applications were analysers for a specific compound or reaction without chromatographic separation. Later, these techniques were applied to the development of specialised selective detectors (Yan 1999). LC-CLND was first described in 1988 (Robbat et al. 1988),

and was subsequently refined and described in its current form in 1992 (Fujinari and Courthaudon 1992). A schematic of the CLND principle is shown in Figure 4. Oxidation of the nebulised LC mobile phase by combustion in a high-temperature furnace converts all nitrogen-containing compounds, except for N_2 , quantitatively into nitric oxide. The dried gas is passed into a chamber where it reacts with ozone, which results in the conversion of nitric oxide to excited-state nitrogen dioxide. The substance subsequently emits chemiluminescent light upon relaxation. The light amplified by the photomultiplier tube is proportional to the moles of nitrogen present in a fraction analysed. All mobile-phase components must be free of nitrogen to keep the baseline noise to a minimum, making acetonitrile, ammonium salts and nitrogenous bases incompatible with CLND detection (Zhang et al. 2008).

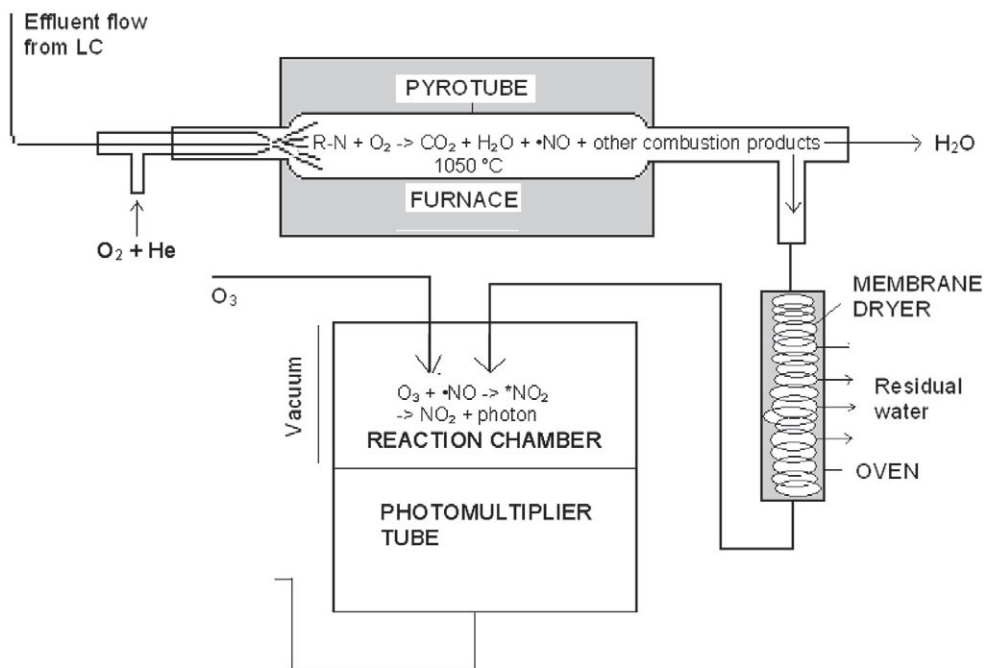


Figure 4. A schematic presentation of a chemiluminescence nitrogen detector (CLND) for liquid chromatography (LC).

Recent improvements in CLND instrumentation, including nebuliser design and ceramic pyrotube, have improved the robustness of the technique and its applicability for routine analysis. In addition, when coupled with an UV detector, CLND can be used to determine relative response factors for any nitrogen-containing compound (Liang et al. 2008). Response ratios between CLND and UV detection (Nussbaum et al. 2002) and between CLND and MS detection (Deng et al. 2004) have been measured in order to extend the concentration range of N-equimolar quantification by LC-CLND.

Originally, CLND was reported to possess an LOD of 0.1 ng nitrogen, a nitrogen/carbon selectivity of 10^7 (Yan 1999) and, over a linear range of 2 orders of magnitude, an equimolar response with $\pm 10\%$ average error for the compounds studied (Taylor et al. 1998). It was also found that the sole exception to equimolar response of the CLND arises from chemical structures containing adjacent nitrogen atoms. A proposed guideline was that the response should be 0 when adjacent nitrogen atoms are connected by a double bond and 0.5 when adjacent nitrogen atoms are connected by a single bond. Later studies have shown that CLND response is highly structure dependent in compounds with adjacent nitrogen atoms connected by a single bond. Substitutions on the nitrogen atoms or atoms nearby in the molecule can increase the CLND response to approach a value higher than the predicted value 0.5 (maximal value 0.82/nitrogen atom). Without substitution, much lower values than predicted (minimal value 0.0-0.08/nitrogen atom) were obtained. Thus, a structurally similar calibration compound should be used for this class of compounds in the quantitative analysis using CLND (Yan et al. 2007).

The CLND linear range of two orders of magnitude was found to be insufficient in some applications of pharmaceutical analysis, when the impurities are present at much lower levels than the surrogate standard. The common practice of direct conversion of area percent to weight per-

cent can result in significant errors using CLND. To increase quantification accuracy, it was proposed that a secondary dilution of the surrogate reference standard solution should be used for the quantification of low-level impurities (Liang et al. 2008).

Single-calibrant quantification by LC-CLND is straightforward in relatively simple materials requiring no extraction, such as combinatorial chemistry library products (Corens et al. 2004, Letot et al. 2005) or nitrogen-containing anions in seawater (Lucy and Harrison 2001). However, there are currently very few LC-CLND applications for biological samples. In the experimental methods presented for rat bile and urine (Taylor et al. 2002), dog plasma and urine (Deng et al. 2004) and microsomal incubations (Edlund and Baranczewski 2004), sample preparation relied on protein precipitation followed by direct LC injection omitting the extraction step. In methods involving protein precipitation, the occasionally necessary freeze-drying may result in losses of volatile analytes. A benefit from an extraction step would be a cleaner chromatographic background and easier sample concentration, but quantification without PRS presumes known extraction recoveries. An SPE method has been reported for the determination of imidacloprid in fruit and vegetables with a relatively constant extraction recovery, but the study utilised CLND solely as a nitrogen-specific detector without taking advantage of the N-equimolar response (Ting et al. 2004).

CLND is a particularly attractive detector in the quantitative analysis of drugs, metabolites and designer drugs in forensic and clinical contexts without PRS, due to its specificity and equimolar response to nitrogen. The LC version of the detector is more useful than the GC version, because it allows detection of a wide range of compounds without derivatisation. A major challenge is the development of sample preparation procedures that can minimise excessive background noise and compensate for varying extraction recoveries.

3. AIMS OF THE STUDY

The aim of this thesis work was to investigate how the qualitative and quantitative analysis of drugs in forensic and clinical contexts may be best performed without the necessity of possessing the respective primary reference standards (PRS).

Specific aims of the studies were:

- I To develop and evaluate an automated LC-TOFMS method for urine drug screening, essentially based on accurate mass measurement and reverse search using a large target database of monoisotopic masses.
- II To add isotopic pattern comparison as a new identification parameter to the LC-TOFMS method.
- III To apply LC-TOFMS to the identification and LC-CLND to the quantification of drugs in seized material as a novel approach for instant substance characterisation without PRS.
- IV To develop an LC-CLND method for the quantification of basic drugs in plasma and whole blood without PRS.
- V To evaluate the feasibility of the LC-CLND method in a pharmacogenetic context by studying plasma tramadol metabolite ratios without PRS.

4. MATERIALS AND METHODS

More detailed descriptions of the materials and methods are presented in the original publications (I-V).

4.1. Reagents

Water was Direct-Q 3 purified (Millipore, Bedford, MA, USA). LC mobile phase components, methanol and acetonitrile, were HPLC grade from Rathburn (Walkerburn, UK). Jeffamine D230 and analytical grade solvents, *n*-butyl chloride and isopropyl alcohol, were from Fluka (Buchs, Switzerland). β -glucuronidase was from Roche (Mannheim, Germany). Other chemicals and reagents were analytical grade from Merck (Darmstadt, Germany), J.T. Baker (Deventer, The Netherlands) and Sigma-Aldrich (Steinheim, Germany). Isolute HCX-5 (100 mg) mixed-mode solid-phase extraction (SPE) cartridges were from International Sorbent Technology (Hengoed, UK).

4.2. Reference standards and materials

Tramadol and its metabolites were a kind gift from Grünenthal GmbH (Aachen, Germany). Caffeine standards were purchased from Ultra Scientific (Wesel, Germany) and from Sigma-Aldrich (Steinheim, Germany). Other drug reference standards were obtained from various pharmaceutical companies. Certified reference serum samples for drugs were purchased from LGC Promochem (Teddington, UK). Reference whole blood samples for drugs were from the Nordquant proficiency test program (Oslo, Norway), involving 13 participants. Pooled blank human plasma was obtained from the Finnish Red Cross Blood Service, and blank whole blood was bovine blood from an abattoir. Blank urine was from various volunteer donors. Seized samples

were obtained from the National Bureau of Investigation, Finland.

4.3. Study subjects

Case urine samples were collected at autopsies for qualitative LC-TOFMS experiments (I, II). In the LC-CLND study involving tramadol (V), blood samples from the volunteer subjects were collected in EDTA tubes one hour after administration of 100 mg of tramadol hydrochloride orally, and plasma was separated by centrifugation.

4.4. Methods

4.4.1. Sample preparation

In LC-TOFMS urine screening experiments (I, II), urine samples of 1 ml were hydrolysed with β -glucuronidase for 2 h at 56°C in a water bath. As an internal standard, 10 μ l of dibenzepin (10 μ g/ml in methanol) was added to the hydrolysed samples. The extraction was performed according to International Sorbent Technology application note IST 1044 A (IST 1997), with minor modifications. The pH of urine samples was adjusted between 5 and 7 with 2 ml of 0.1 M phosphate buffer (pH 6). The SPE cartridge was conditioned with 2 ml of methanol and equilibrated with 2 ml of water and 3 ml of 0.1 M phosphate buffer (pH 6). After sample addition, the cartridge was washed with 1 ml of phosphate buffer and with 1 ml of 1 M acetic acid, and after both washing steps, dried under full vacuum for 5 min. The acidic-neutral fraction was eluted with 3 ml of ethyl acetate-hexane (25+75, v/v). The cartridge was dried for 2 min and washed with 3 ml of methanol. After drying for 2 min, basic drugs were eluted with 3 ml of ethyl acetate-ammonium hydroxide (98+2, v/v). After extraction, the acidic and basic fractions were com-

bined and evaporated to dryness at 40°C. The dried sample was reconstituted with 150 µl of acetonitrile-0.1% formic acid (1+9, v/v).

For the analysis of seized street drug samples (III), the material, which in all cases was in solid form, was homogenised and 1-4 mg was dissolved in methanol to obtain a stock solution of 1 mg/ml. For identification, the stock solution was diluted 1:100 with methanol-0.1% formic acid (1+9, v/v). If MS response was low with the dilution of 1:100, the stock solution was used for identification instead. For quantification, three dilutions were made separately from the stock solution: 1:2 with methanol-0.1% formic acid (1+1, v/v), and 1:10 and 1:100 with methanol-0.1% formic acid (3+7, v/v).

For the LC-CLND quantification of drugs in plasma and whole blood (IV) to a 5 ml sample, 2 ml of 1 M TRIS buffer (pH 11) was added. pH was adjusted to 10 by 5 M sodium hydroxide. After addition of 10 ml of *n*-butyl chloride-isopropanol (98+2, v/v), the sample was shaken for 30 min in a rotor shaker. Following phase separation by centrifugation (10 min at 4000 rpm), 7.5 ml of the organic layer was transferred into another test tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted with 100 µl of methanol-0.1% formic acid (1+1, v/v), and after vortexing and centrifugation, the supernatant was transferred into an autosampler vial for LC-CLND analysis.

For the LC-CLND quantification of tramadol and its metabolites (V), 10 µl of the internal standard solution (0.1 mg/ml of dextropropoxyphene in methanol) was added to a 5 ml sample to yield a concentration of 200 µg/l. The extraction procedure was similar to that in study IV with the following exceptions: the volume of *n*-butyl chloride-isopropanol (98+2, v/v) was 7 ml, and 5.5 ml of the organic layer was collected. The evaporated residue was reconstituted with 75 µl of methanol-0.1% formic acid (2+8, v/v).

In all quantitative studies, caffeine stock solutions were diluted with water to obtain the calibration standards (III, IV, V).

4.4.2. LC-TOFMS

The mass analyser in studies I and III was an Applied Biosystems (Framingham, MA, USA) Mariner TOF mass spectrometer, equipped with a PE Sciex (Concord, ON, Canada) Turbolon Spray source and a 10-port switching valve. The nebuliser gas (N₂) flow was 0.7 l/min, the curtain gas (N₂) flow was 1.2 l/min, and the heater gas (N₂) flow was 8 l/min. The spray tip potential of the ion source was 5.5 kV, and the heater temperature was 350°C. Spectrum acquisition time was 2 s, and the *m/z* range recorded was 100-750. Daily instrument tuning and three-ion mass scale calibration was performed with 1.0 µg/ml Jelfamine D-230 solution in acetonitrile-0.1% formic acid (1+1 v/v) by infusion injection. The theoretical exact *m/z* values for the calibration ions were 191.17544, 249.14731, and 317.25917, and a minimum resolution of 5,000 was used in the calibration. Automated post-run internal mass-scale calibration of individual samples was performed by injecting the calibration solution in the beginning of each run via a 10-port switching valve equipped with a 20 µl loop. The liquid chromatograph in the LC-TOFMS system was an Agilent Technologies (Waldbronn, Germany) 1100 series instrument, comprising a vacuum degasser, autosampler, binary pump, column oven and diode array detector (I, III).

In the isotopic pattern experiments (II), the mass analyser was a Bruker Daltonics (Bremen, Germany) micrOTOF mass spectrometer equipped with an ESI source and a 6-port divert valve. The instrument was operated in positive ion mode using an *m/z* range of 50-800. The capillary voltage of the ion source was set at 4,500 V and capillary exit at 90 V. Nebuliser gas flow was 1.6 bar and dry gas flow 8 l/min. Drying gas temperature was set at 200°C. Transfer time of the source was 38 µs and hexapole RF was 75.0 Vpp. Summation of spectra was 10000. Instrument calibration was performed externally prior to each sequence with sodium formate solution, consisting of 10 mM sodium hydroxide in isopropanol-0.2% formic acid (1+1, v/v). The theoretical exact *m/z* of the calibration ions were 158.9464, 240.9671, 362.9263,

430.9138, 498.9011 and 566.8882. Automated post-run internal mass-scale calibration of individual samples was performed by injecting the calibrant at the beginning and at the end of each run via a 6-port divert valve equipped with a 100 μ l loop. Calibration was performed based on calibrant injection at the beginning of the run. The liquid chromatograph in the LC-TOFMS system was an Agilent Technologies (Waldbronn, Germany) 1100 series instrument, comprising a vacuum degasser, autosampler, binary pump, and column oven.

Chromatographic separation in both LC-TOFMS systems was performed with a Phenomenex (Torrance, CA, USA) Luna C-18(2) 100 \times 2 mm (3 μ m) column and a 4 \times 2 mm precolumn in gradient mode at 40°C. The flow rate was 0.3 ml/min. The mobile phase components were 5 mM ammonium acetate in 0.1% formic acid and acetonitrile. The proportion of acetonitrile was increased from 10% to 40% in 10 min, to 75% in 13.50 min, to 80% in 16 min, and held at 80% for 5 min. Equilibrium time was 6 min and injection volume was 10 μ l.

4.4.3. LC-CLND

LC-CLND analysis was performed with a Hewlett-Packard (Agilent) 1090 series liquid chromatograph equipped with an autosampler, three-

channel gradient pumping system, column oven and diode array detector. The nitrogen-specific detector was an Antek (Houston, TX, USA) 8060 CLND. The detector was interfaced with a computer using an HP (Agilent) analog to a digital converter. LC-CLND data were processed using HP Chem Station A.06.01 software (Agilent).

For the CLND analysis, oxygen flow was 250 ml/min, helium 50 ml/min and make-up helium 50 ml/min. Ozone flow was 25 ml/min, and furnace temperature was 1050°C. The photo multiplier tube voltage was set at 750 V and the amplification factor was 25.

External calibration in all quantitative studies was performed at the beginning of the data acquisition sequence with caffeine standards, using calibration points at 0.75, 1.0, 1.5, 3.0, 10 and 30 ng of nitrogen per injection. The curve fit was linear with $R^2 > 0.997$.

LC separation was performed in gradient mode at 40°C using a Phenomenex (Torrance, CA, USA) Luna C-18(2) 100 \times 2 mm (3 μ m) column, equipped with a 4 \times 2 mm precolumn (III, V) and a Phenomenex (Torrance, CA, USA) Gemini C-18(2) 150 \times 2 mm (3 μ m) column, equipped with a 4 \times 2 mm precolumn (IV). Mobile-phase components were 0.1% formic acid and methanol. Diode array detector signal was recorded at 230 nm, and peak controlled spectra were recorded at 210–400 nm (III, IV, V).

5. RESULTS AND DISCUSSION

The main results of the studies are described in this section, more detailed results can be found in the original publications I-V.

5.1. Urine drug screening by LC-TOFMS

5.1.1. High-throughput screening based on accurate mass (I)

Gergov et al. reported a concept for urine drug screening by positive electrospray ionisation LC-TOFMS with an automated target database search based on molecular formulae, relying on the assumption that tentative identification of drugs in urine is viable without PRS by use of exact monoisotopic masses and metabolite patterns from the literature (Gergov et al. 2001). The present study evaluates this LC-TOFMS (Applied Biosystems Mariner) based screening methodology to the full with a series of urine samples taken at autopsy and shows its scope and limitations in forensic toxicology practice. The method relied on a large target database of exact monoisotopic masses representing the molecular formulae of reference drugs and their metabolites. Identification by reverse search was based on matching sample components' measured parameters with those in the target database, including accurate mass and RT if available. Data post-processing software was developed for automated reporting of findings in an easily interpretable form. The screening method was validated by measuring the precision of LC RT and LODs for representative compounds. In addition, the findings from autopsy urine samples were compared with those obtained by a GC-MS reference method.

A target database was constructed, including 637 monoisotopic masses for the protonated molecules of toxicologically relevant therapeutic drugs, drugs-of-abuse, metabolites, design-

er drugs and pesticides. In addition to monoisotopic mass, the database included the following data for each compound: name of compound, molecular formula, RT if available, and a 3-5-digit compound code. The code specified the compound group, the number of compounds in the group, and the ordinal number of the compound in the group. The chemical and pharmacological properties of the compounds included in the database varied greatly, but the majority of the entries represented basic drugs that could be obviously ionised with ESI in the positive mode. For 392 compounds, a PRS was available to the authors and the corresponding LC RT could be determined. The remaining database entries involved mainly drug metabolites reported in the scientific literature, and for these entries, only the exact mass without an RT was included in the database. Both RT and MS ionisation data were recorded by running mixed reference standard solutions containing 8-10 substances each at a concentration of 1 µg/ml in acetonitrile-0.1% formic acid (1+9, v/v). Seven substances were excluded from the database, i.e. ethyl parathion, methyl parathion, cyclothiazide, dichlorprop, MCPA, ibuprofen and γ -hydroxy butyrate, because they did not ionise under the conditions used. Nine substances – acetazolamide, apronamide, chlorpropamide, chlorthalidone, felodipine, phenytoin, primidone, salicylamide and sulthiame – had low ionisation efficiency, and thus high concentrations were required to obtain sufficient ion abundance.

To study the precision of RT and RRT, three repetitive runs were performed within one week, and the set of three runs was repeated two times at one-month intervals. Relative standard deviation (RSD) values for RT and RRT were calculated from these nine parallel runs. For RRT calculations, dibenzepin was added to each reference standard solution as an internal standard. Mean RSD for RT and RRT was 0.50% and 0.65%, respectively. Repeatability of RT and RRT proved to be of the same magnitude, therefore RT was

chosen to be the LC identification parameter as it is simpler to manage. Recalibration of RT values was required at approximately six-month intervals, mainly after changing to a new chromatographic column. Although RRT use could have facilitated the recalibration procedure, more than one internal standard might have been needed due to the large polarity scale of the analytes and the batch-to-batch variability of sorbent materials.

Table 1 shows the LOD for 90 representative substances. LOD was determined by spiking

blank urine samples with drugs and metabolites in decreasing concentrations, starting with an initial concentration of 0.1 mg/l. If a compound was not detectable at 0.1 mg/l, the concentration was increased until a sufficient response was obtained. Three parallel analyses were performed at the LOD level, and a compound was considered detected if it was reported in the results report in all three cases. The criteria for reporting included a 30 ppm mass tolerance, a ± 0.2 min RT window and a minimum peak area count of 500.

Table 1. Limits of detection (LOD) and retention times (RT) for 90 compounds in urine by LC-TOFMS

Compound	RT (min)	LOD (mg/l)
Acebutolol	7.42	0.01
Alprenolol	10.51	0.01
Amiodarone	16.33	10.0
Amitriptyline	12.99	0.02
Amphetamine	3.12	0.1
Atenolol	1.59	0.1
Benzoylcegonine	6.63	0.5
Betaxolol	10.78	0.01
Bisoprolol	9.61	0.02
Buprenorphine	11.64	0.2
Carbamazepine	13.12	0.02
Carvedilol	12.36	0.1
Celiprolol	8.88	0.02
Chloroquine	2.94	0.2
Chlorpromazine	13.59	0.1
Chlorprothixene	13.81	0.5
Cisapride	11.98	0.5
Citalopram	11.44	0.02
Clomipramine	13.97	0.05
Clonazepam	14.43	0.2
Clonidine	2.63	0.01
Clozapine	10.73	0.05
Cocaine	8.36	0.02
Codeine	2.47	0.05
Dextropropoxyphene	12.99	0.02
Diazepam	16.04	0.01
Diltiazem	11.74	0.02
Dixyrazine	13.55	0.1
Doxepin	11.51	0.02
Ethylmorphine	5.11	0.05
Flunitrazepam	15.04	0.05
Fluoxetine	13.60	0.2
Flupentixol	14.61	2.0
Fluvoxamine	12.83	0.05
Glibenclamide	16.80	0.01
Glipizide	15.01	0.1
Hydrocodone	3.94	0.1
10-hydroxycarbamazepine	9.78	0.1
Indomethacin	16.79	0.5
Ketoprofen	15.44	1.0
Levomepromazine	13.06	0.05

Lorazepam	14.29	0.2
LSD	9.25	0.02
Maprotiline	12.86	0.1
MDMA (ecstasy)	4.18	0.05
Methadone	13.11	0.01
Metamphetamine	3.78	0.01
Metoprolol	7.52	0.02
Mianserine	11.12	0.02
Midazolam	11.17	0.05
Moclobemide	5.89	0.01
6-monoacetylmorphine (MAM)	3.63	0.05
Morphine	1.49	0.2
Nicotine	1.07	0.5
Nizatidine	1.47	0.02
Norcitalopram	11.25	0.05
Norclomipramine	13.78	0.1
Nordiazepam	15.04	0.1
Norflunitrazepam	13.81	0.2
Normianserine	11.00	0.05
Nortriptyline	12.77	0.05
Olanzapine	3.29	0.05
Orphenadrine	12.10	0.05
Oxazepam	13.91	0.1
Oxprenolol	9.06	0.1
Paracetamol	2.44	>50
Paroxetine	12.36	0.5
Perphenazine	13.62	0.5
Phenazone	7.32	0.05
Phencyclidine	10.00	0.01
Phenytoin	13.27	>50
Pindolol	4.47	0.5
Practolol	1.91	0.05
Promazine	12.17	0.1
Propranolol	10.35	0.01
Quinine	7.36	0.1
Ranitidine	1.73	0.05
Risperidone	9.63	0.02
Selegiline	6.27	0.1
Sotalol	1.69	0.5
Sulpiride	1.96	0.01
Thioridazine	14.51	0.5
Timolol	7.11	0.02
Tramadol	7.45	0.01
Trimipramine	13.25	0.1
Venlafaxine	9.42	0.01
Verapamil	13.03	0.02
Warfarin	16.13	0.02
Zolpidem	9.02	0.01
Zopiclone	7.34	0.2

A mixed mode SPE sorbent combining cation exchange and hydrophobic interaction was chosen for extraction. A short carbon chain C4 provided the cleanest analytical background, yet good recoveries. LOD in urine was ≤ 0.1 mg/l for 66 of the 90 compounds studied, and only five compounds had an LOD ≥ 1 mg/l. Paraceta-

mol and phenytoin were not detected even at a concentration of 50 mg/l, because of their low SPE extraction recovery and poor ionisation in the positive mode. For acidic compounds, negative ionisation would presumably provide lower LODs. However, the concentrations of acidic drugs in biofluids are normally higher than those

of basic drugs (Baselt 2004), and consequently, the present method also allows detection of many acidic drugs. Generally, the LODs obtained with the present method were higher than those reported with MRM target analyses by LC-MS (Van Bocxlaer et al. 2000), but at the same level as in other screening applications (Gergov et al. 2000, Thevis et al. 2001).

Fifty authentic autopsy urine samples were analysed by the LC-TOFMS method, and the results were compared with a reference GC-MS method applying comprehensive commercial spectrum libraries. Figures 5a and 5b show an LC-TOFMS total ion chromatogram (TIC) and a corresponding results report list for an autopsy urine sample. Compounds marked bold in Figure 5b are considered as true positive findings in the report. The number of true positives, false positives, false negatives and unidentified compounds (not included in the database) by LC-TOFMS are shown in Figure 6. For compounds with an RT available, this classification was based on the following criteria. Entries in the report

with molecular mass of ≥ 200 Da and mass error ≤ 20 ppm were considered true positive findings if a parent drug and at least one metabolite were reported. For entries with molecular mass < 200 Da, the mass error tolerance was 30 ppm. Entries were considered as false positive findings, if the mass criteria were met but without metabolites being reported and with a negative GC-MS screen. The total number of compounds identified by LC-TOFMS was higher than by GC-MS. However, some acidic compounds, such as ibuprofen and valproic acid, were not detected by LC-TOFMS as they were not ionisable in positive ESI, and therefore they were not included in the database. Seven caffeine findings were missed by LC-TOFMS, mainly due to the low mass and poor peak shape of caffeine, resulting in a mass error higher than 30 ppm. The false positive findings, such as meclozine, amitriptyline and dextropropoxyphene, were apparently endogenous components from autopsy urine samples with unresolved adjacent molecular mass.

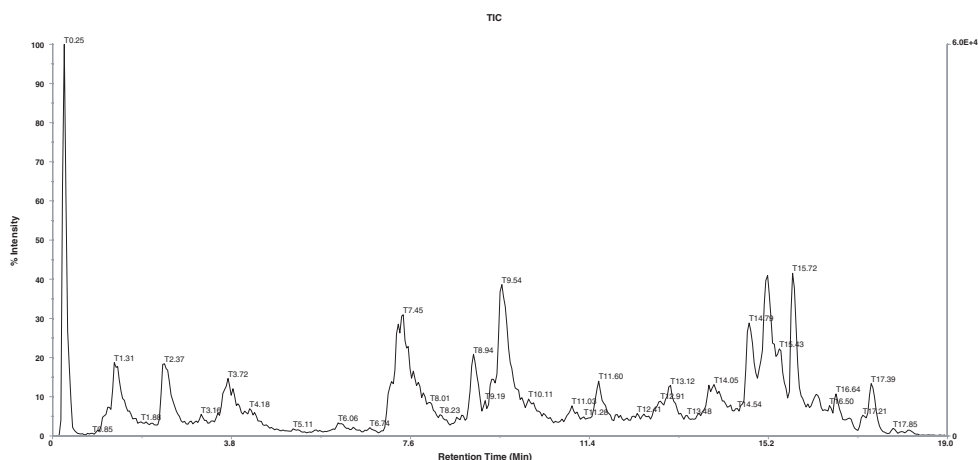


Figure 5a. A total ion chromatogram of a solid-phase extracted autopsy urine sample acquired by LC-TOFMS.

Sample ID: 4551
 Acquisition Date: Sep 02 02:02:00 2008
 Report Date: 9/2/2008 09:39

Mass List Used: C:\Mariner\Program\screening\TOFseu070307.xls
 Set File Used: C:\Mariner\Program\screening\APScreen.set

Compound	RT [min]	RT exp [min]	d RT [min]	Err [ppm]	Err [mDa]	Peak Area	Mass Found	Ref. Mass	Formula	Reg. No.
NORDIAZEPAM	15,08	15,16	-0,08	-9,6	-2,6	2349,4	271,0659	271,0633	C15H11N2OCl	154
OXAZEPAM	14,05	14,01	0,04	-8,2	-2,3	5041,9	287,0605	287,0582	C15H11N2O2Cl	155
DIAZEPAM	16,07	16,17	-0,1	-12,6	-3,6	908,4	285,0825	285,0789	C16H13N2OCl	1341
TEMAZEPAM	15,18	15,31	-0,13	-4,6	-1,4	11083,5	301,0752	301,0738	C16H13N2O2Cl	1343
CAFFEINE	4,22	4,2	0,02	-17,1	-3,3	4316,5	195,091	195,0877	C8H10N4O2	1451
CODEINE	2,37	2,29	0,09	-3,4	-1	16648,6	300,1604	300,1594	C18H21NO3	1831
NORCODEINE	2,16	2,06	0,11	-19,1	-5,5	562,2	286,1492	286,1438	C17NH19O3	1832
4-HYDROXYPROPANOLOL	12,63	0	12,63	-12,7	-3,5	1318,2	276,1629	276,1594	C16H21NO3	2144
NORHYDROCODONE	2,16	0	2,16	-19,1	-5,5	562,2	286,1492	286,1438	C17H19NO3	2932
HYDROXYCLOMIPRAMINE	11,03	0	11,03	-0,9	-0,3	633,1	331,1575	331,1572	C19H23N2OCl	3633
NORCLOBAZAM	14,05	0	14,05	-8,2	-2,3	5041,9	287,0605	287,0582	C15N2H11O2Cl	3832
P-HYDROXYNOREPHEDRINE	1,31	0	1,31	21,3	3,6	973,3	168,0983	168,1019	C9H13NO2	3933
NORALPRENOLOL	4,08	0	4,08	-17	-4	2127,2	236,1685	236,1645	C14H21NO2	7633
P-HYDROXYPHENYTOIN	14,86	0	14,86	25,7	6,9	2029,7	269,0852	269,0921	C15H12N2O3	7942
M-HYDROXYPHENYTOIN	14,86	0	14,86	25,7	6,9	2029,7	269,0852	269,0921	C15H12N2O3	7943
3,4-DIHYDRODIHYDROXYPHENYTOIN	12,95	0	12,95	-5,8	-1,7	1002,5	287,1043	287,1026	C15H14N2O4	7944
TRAMADOL	7,41	7,37	0,04	5,1	1,3	52180	264,1945	264,1958	C16NH25O2	8861
O-DESMETHYLTRAMADOL	3,72	3,56	0,16	-3,7	-0,9	6501,2	250,1811	250,1802	C15H23NO2	8862
NORTRAMADOL	7,59	7,48	0,11	-19,3	-4,8	2236	250,185	250,1802	C15H23NO2	8863
O-DESMETHYLNORTRAMADOL	4,08	3,88	0,2	-17	-4	2127,2	236,1685	236,1645	C14H21NO2	8864
NORMEDIAZEPAM	11,7	0	11,7	-21,4	-5,5	795,5	257,0895	257,084	C15H13N2Cl	9466
HYDROXYMETHYLTOLBUTAMIDE	16,07	0	16,07	28	8	1772,3	285,0824	285,0904	C12H16N2O4S	9533
DINORVENLAFAXINE	3,72	0	3,72	-3,7	-0,9	6501,2	250,1811	250,1802	C15H23NO2	9844
DINORVENLAFAXINE	7,59	0	7,59	-19,3	-4,8	2236	250,185	250,1802	C15H23NO2	9844
NORATROPINE	12,63	0	12,63	-12,7	-3,5	1318,2	276,1629	276,1594	C16H21NO3	10432
ZOLPIDEM	8,94	8,92	0,02	0,4	0,1	7891,4	308,1756	308,1757	C19H21N3O	10941
2-QUINIDONE	3,4	0	3,4	-15,7	-5,4	1409,4	341,1913	341,186	C20H24N2O3	12132
3-HYDROXYQUINIDINE	3,4	0	3,4	-15,7	-5,4	1409,4	341,1913	341,186	C20H24N2O3	12133
QUININE	7,2	7,17	0,03	-8,8	-2,8	7547	325,1939	325,1911	C20N2H24O2	12231
3-HYDROXYQUININE	3,4	0	3,4	-15,7	-5,4	1409,4	341,1913	341,186	C20H24N2O3	12232
2-HYDROXYQUININE	3,4	0	3,4	-15,7	-5,4	1409,4	341,1913	341,186	C20H24N2O3	12233
NORPROPAFENONE	2,37	0	2,37	-3,4	-1	16648,6	300,1604	300,1594	C18H21NO3	12933
INDOMETHACIN	16,85	16,93	-0,08	5,1	1,8	785,4	358,0822	358,0841	C19NH16O4Cl	13231
HALOPERIDOL REDUCED	15,72	0	15,72	6,3	2,4	4908,6	378,1607	378,1631	C21H25NO2ClF	13654
DINORACETYLMETHADOL	7,23	0	7,23	28,1	9,2	1260	326,2023	326,2115	C21H27NO2	15243
3-METHYLNORFENTANYL	1,38	0	1,38	-10,2	-2,5	6546,6	247,183	247,1805	C15H22N2O	15522
METHOXYTADALAFIL	16,25	0	16,25	26,1	10,2	1107,9	392,1502	392,1605	C22H21N3O4	16433
RIVASATIGMINE METAB2	11,03	0	11,03	-22,4	-3,7	882,1	164,1107	164,107	C10H13NO	17033
NORDIHYDROCODEINE	14,79	0	14,79	-4,2	-1,2	10135,7	288,1606	288,1594	C17H21NO3	17222
NORSIBUTRAMINE	8,94	0	8,94	12	3,2	705,1	266,1638	266,167	C16H24ClN	17432
2C-O-4	11,81	0	11,81	-22,8	-5,5	558,1	240,1649	240,1594	C13H21O3N	n/a
IP	11,81	0	11,81	-22,8	-5,5	558,1	240,1649	240,1594	C13NH21O3	n/a
EMM	11,81	0	11,81	-22,8	-5,5	558,1	240,1649	240,1594	C13H21O3N	n/a
MEM	11,81	0	11,81	-22,8	-5,5	558,1	240,1649	240,1594	C13NH21O3	n/a
DIBENZEPIN	9,4	9,31	0,09	-14,7	-4,4	4058,2	296,1801	296,1757	C18H21N3O	n/a
BISOPROLOL	9,54	9,52	0,02	-6,1	-2	44628,6	326,2346	326,2326	C18NH31O4	n/a
AJMALINE	8,01	0	8,01	-0,1	0	565,4	327,2067	327,2067	C20H26N2O2	n/a
AJMALINE	9,54	0	9,54	-0,1	0	2509	327,2067	327,2067	C20H26N2O2	n/a
MECLOZINE	15,72	15,56	0,16	22,4	8,8	2069,9	391,1848	391,1936	C25H27N2Cl	n/a

Figure 5b. A results report list generated by the analysis macro program for the autopsy urine sample of Figure 5a. Entries in bold are considered true positive findings.

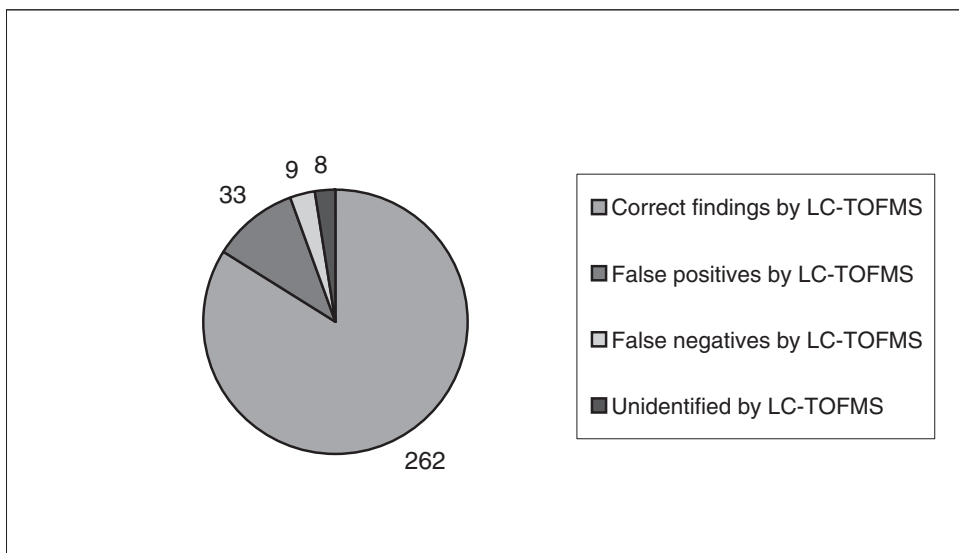


Figure 6. Compounds detected by LC-TOFMS and compared by GC-MS from 50 successive autopsy urine samples. Unidentified compounds are those not included in the LC-TOFMS database.

Identification based solely on accurate mass at the present level of mass accuracy was considered insufficiently reliable, allowing only tentative identification of metabolites without a PRS. However, the combination of accurate mass, metabolite pattern, and available RTs proved to be feasible. The LC-TOFMS method described has been in daily routine use for four years, and all parts of the procedure, from sample preparation to data analysis, have complied with the high-throughput screening concept.

5.1.2. The effect of isotopic pattern in screening analysis (II)

The effect of isotopic pattern determination in combination with accurate mass was evaluated for identification in LC-TOFMS screening. The principle of the LC-TOFMS method was similar to that described in the previous study (I), but a new generation TOFMS analyser (Bruker microTOF) with improved performance was used. The instrument allowed measuring a numerical value for the isotopic pattern match, SigmaFit™, as a new identification parameter together with the accurate mass on a routine basis. Two identification procedures were compared with five repre-

sentative autopsy urine samples: combination of accurate mass and isotopic pattern, and accurate mass only. The number of entries in the result report list and the proportion of true positive findings were studied.

The database was based on that used in study I, but it was extended to include 735 entries. From the data acquired by LC-TOFMS, the automated database search reported hits within a selected retention time window, peak area, mass tolerance and isotopic pattern match. A post-processing script created extracted ion chromatograms (EIC) of the expected MH^+ ions for each compound of the database within a very narrow m/z window ($\pm 3 \times 10^{-3}$). On these, trace peak detection was applied, and a MS spectrum was created for each chromatographic peak. Each MS spectrum was associated with a distinct substance in the database and a SigmaFit (isotopic pattern match) was calculated. This included generation of a theoretical isotope pattern and calculation of a match factor based on the deviation from the measured ion abundances. Final sorting and scoring of the result list was performed by an MS Excel script based on RT, mass error tolerance, SigmaFit and peak area. An example of measured and calculated isotopic patterns is shown in Figures 7a and 7b.

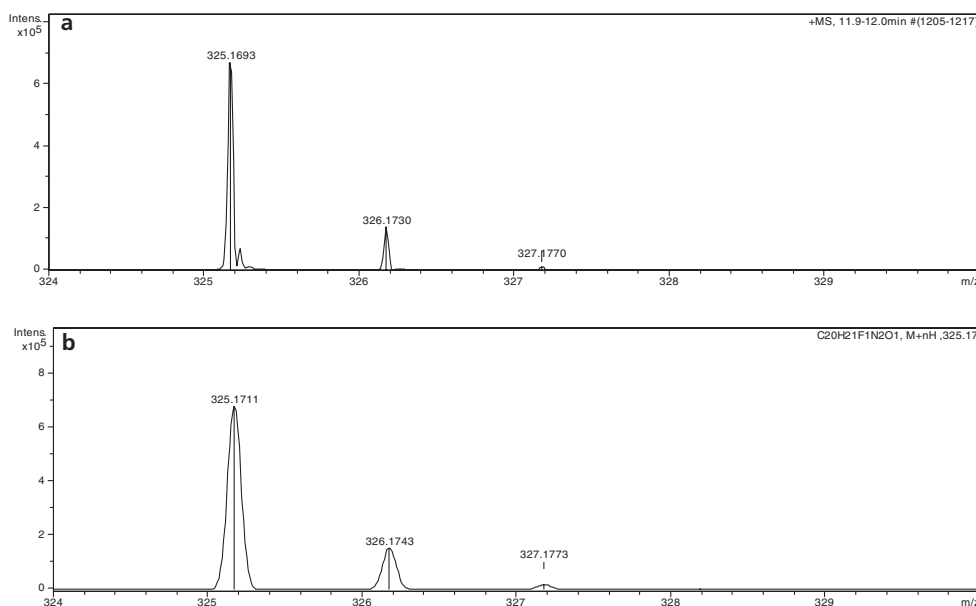


Figure 7a ja 7b. Measured (a) and calculated theoretical (b) isotopic pattern of citalopram.

Five parallel runs of each solid-phase-extracted autopsy urine samples were performed by LC-TOFMS. For data handling, two data processing software settings were used. In the first procedure (procedure A), identification was based on both accurate mass and isotopic pattern, with a SigmaFit upper limit set at 0.03 and a mass error tolerance set at 10 ppm. In the second procedure (procedure B), identification was based on accurate mass only, with a SigmaFit upper limit set at 1.0 and a mass error tolerance set at 10 ppm. In both procedures, the minimum area count was set at 50,000 and a retention time window of ± 0.3 min was used for those compounds for which retention time was available in the database. The entries in the results report lists by procedure A and procedure B were compared, and the findings were classified as true positive, false positive and false negative findings. The reference list of true positive findings was based on the laboratory's established methods of investigation and case background information.

The mean SigmaFit value for the true positive findings was 0.0066 (median 0.0051). The selected SigmaFit tolerance value of 0.03 was

exceeded by only one true positive entry, representing hydroxalprazolam. This was a false negative by procedure A. In this case the SigmaFit value was as high as 0.5 in all five parallel runs because of a co-eluting matrix compound with an adjacent molecular mass. The mean of the mass error absolute values was 2.51 ppm (median 2.17 ppm), corresponding to 0.65 mDa (median 0.60 mDa), and the range was from -4.90 to 9.80 ppm. The mass accuracy or SigmaFit was not affected by ion abundance or the concentrations of the detected compounds.

TIC and EIC for a urine sample are shown in Figures 8a and 8b, respectively, and the corresponding results report list is presented in Figure 8c. The compounds highlighted by the software are those for which RT, SigmaFit and mass error were within the pre-selected limits. The compound codes facilitated the interpretation of the findings by connecting parent compounds with their metabolites (see Materials and Methods).

The lengths of the results report lists by procedure A and procedure B are compared in Figures 9a and 9b. The false positive entries were mainly metabolites of compounds for which an

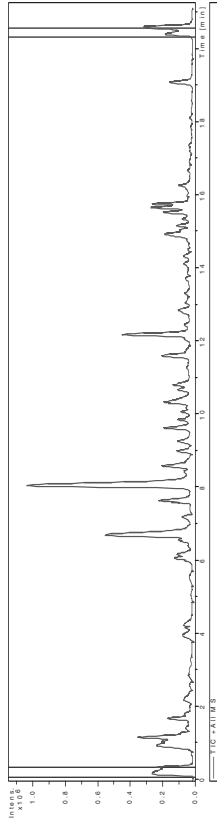


Figure 8a. A total ion chromatogram of a solid-phase extracted autopsy urine sample acquired by LC-TOFMS.

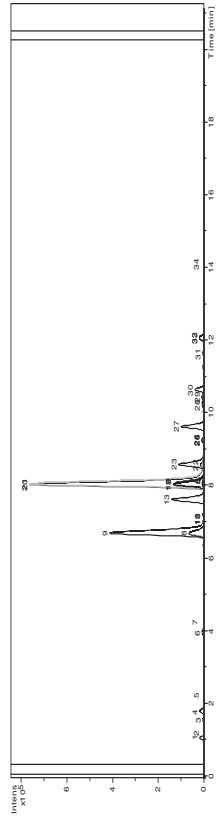


Figure 8b. An extracted ion chromatogram generated by the post-processing software, showing the target compounds found in the autopsy urine sample of Figure 8a.

m/z (theo)	err [ppm]	sigma	meas rt	rt (theo)	delta rt	substance	code	PseudoM	formula	area	Cmpd
195.0877	-0.44	0.0017	4.303	4.1	0.2	CAFFEINE	1451	M++	C8H11N4O2	231966	7
260.1645	0.39	0.0095	10.667	10.49	0.18	PROPRANOLOL	2141	M++	C16H22N1O2	303507	30
276.1694	0.72	0.0066	7.113	0	7.11	4-HYDROXYPROPRANOLOL	2144	M++	C16H22N1O3	61809.5	10
152.0706	-5.71	0.0043	2.285	2.33	-0.05	PARACETAMOL	5022	M++	C8H10N1O2	145553	5
266.1652	1.46	0.0124	8.067	7.95	0.12	MIRTAZAPINE	8921	M++	C17H20N3	7670258	20
252.1495	1.06	0.0124	7.646	0	7.65	NORMIRTAZAPINE	8922	M++	C16H18N3	1195411	13
276.1694	0.72	0.0066	7.113	0	7.11	NORATROPINE	10432	M++	C16H22N1O3	61809.5	11
306.17	1.70	0.0076	8.049	0	8.05	ATROPINE-N-OXIDE	10433	M++	C17H24N1O4	62961.8	14
308.1757	0.12	0.0032	9.268	9.11	0.16	ZOLPIDEM	10941	M++	C19H22N3O1	92475.3	24
427.214	0.34	0.0130	9.269	0	9.27	7-HYDROXYRISPERIDONE	14353	M++	C23H28F1N4O3	87417.6	25
427.214	0.34	0.0130	9.269	0	9.27	9-HYDROXYRISPERIDONE	14354	M++	C23H28F1N4O3	87417.6	26
296.1757	3.13	0.0248	9.628	9.41	0.22	DIBENZEPIN	99999	M++	C18H22N3O1	717680	27

Figure 8c. A results report list for the autopsy urine sample of Figures 8a and b generated by the post-processing software. The highlighted entries are those for which retention time, SigmaFit and mass error were within the pre-selected limits.

RT was not available in the database due to a lack of reference substances. The number of false positives and the capability of detecting substances at trace levels is a compromise, and in practical work, it is important to optimise the sensitivity of the LC-TOFMS method. Procedure A, based on accurate mass and isotopic pattern, produced 61% true positive findings. Procedure B, based on accurate mass only, produced more false positive findings and the proportion of true positive findings was 49%. The false negative findings in procedure A represented hydroxy-alprazolam (see above) and dibenzepin in both procedures. The latter was due to a co-eluting doxepin *N*-oxide peak, possessing an molecular

mass adjacent to dibenzepin.

A mass tolerance of 10 ppm and a SigmaFit upper limit of 0.03 proved to be appropriate values for identification. Isotopic pattern match clearly decreased the number of false positive entries on the result report lists. This was observed especially with concentrated and putrefied urines, but also with relatively clean samples. Very constant SigmaFit and mass error values were obtained for true positive findings. The present LC-TOFMS method allowed the use of a narrower mass window than in the original method (I), in which a mean mass error was 7 ppm and a mass tolerance of 30 ppm was used.

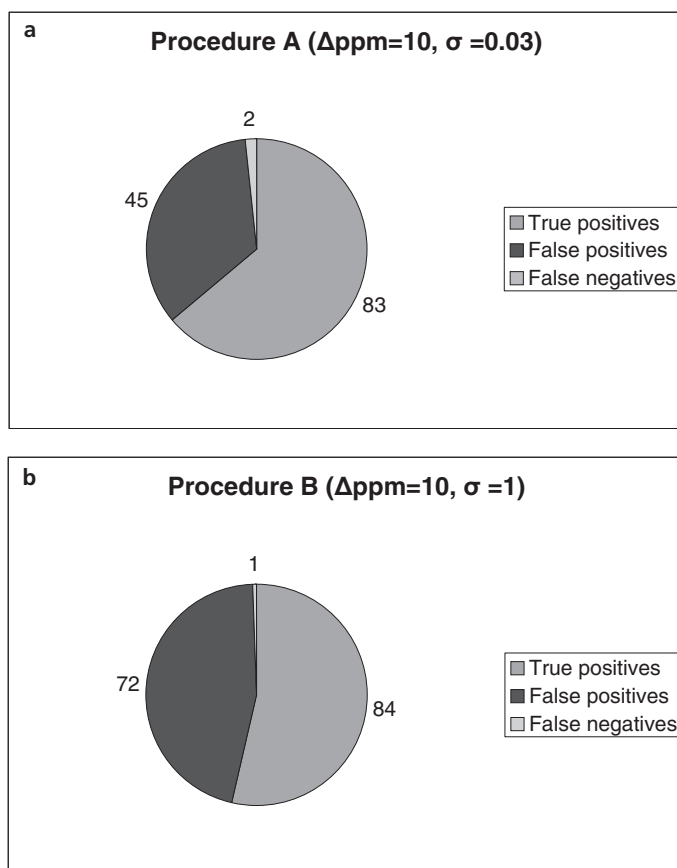


Figure 9a ja 9b. Lengths of the results report lists by LC-TOFMS using procedure A, utilising SigmaFit and accurate mass (a), versus procedure B, utilising accurate mass only (b). The false positive entries were mainly metabolites for which retention time was not available in the database.

Isotopic pattern determination is not a new invention; it has been previously used in substance identification already in 1980s by using low resolution MS (Kavanagh 1980). Isotopic pattern has proven useful for confirming compound identity and facilitating chemical structure characterisation by mass spectrometry, because it is information-rich and almost independent of instrument type and ionisation technique (Rockwood et al. 2003). The present thesis is the first to utilise isotopic pattern determination in comprehensive LC-TOFMS screening analyses based on a target database search. Use of isotopic patterns clearly improved the performance of the method, but false positives report entries were still produced for compounds for which PRS were not available. Hence, the combination of accurate mass and isotopic pattern provides a method for tentative identification, which should then be confirmed with other methods.

5.2. Analysis of street drugs (III)

Twenty-one seized street drug samples were analysed qualitatively by LC-TOFMS (Mariner), using the method described earlier (I), and quantified by LC-CLND using caffeine as single secondary standard. The sample preparation procedure was kept simple and rapid, comprising only dilution. The results were compared with those obtained by accredited reference methods (see original publication). The LC-TOFMS database consisted of 735 compounds, including therapeutic drugs, drugs-of-abuse, metabolites and designer drugs. Identification of designer drugs was based on accurate mass only, because the corresponding PRS were not available. Table 2 shows the results obtained by LC-TOFMS and LC-CLND, compared with those of the reference methods.



The logo of the Department of Forensic Medicine designed by Professor Kari Suomalainen.

Table 2. Identification of seized samples by LC-TOFMS and quantification by LC-CLND

Sample	Compounds identified by LC-TOFMS	Mass error by LC-TOFMS (ppm)	Compounds identified by reference methods	C (%) by LC-CLND	RSD (%) By LC-CLND	C (%) by reference methods	Relative difference (%) between methods
1	amphetamine	-14.8	amphetamine	23	4.3	22	4.5
2 ^a	heroin	-8.6	heroin	2.5	16	2.4	4.2
	6-monoacetylmorphine (MAM)	-5.7	6-monoacetylmorphine (MAM)	4.4	5.8	4.9	-10
	amphetamine	-24	amphetamine	3.1	2.9		
	chloroquine	-9.9	chloroquine				
			desethylchloroquine ^b				
			acetylcodeine ^b				
3	amphetamine	-9.0	amphetamine	49	2.5	42	17
4 ^a	amphetamine	-7.6	amphetamine	8.1	14	8.8	-8.0
	cocaine	5.6	cocaine	21	2.2	24	-13
	lidocaine	-2.5	lidocaine				
			piracetam ^b				
			cinnamoylcocaine ^b				
5	5-methoxy- <i>N,N</i> -dimethyltryptamine	-5.1	5-methoxy- <i>N,N</i> -dimethyltryptamine	120	4.7		
6	5-methoxytryptamine	1.2	5-methoxytryptamine	97	0.12		
7	amphetamine	-3.2	amphetamine	69	0.51		
8	4-iodo-2,5-dimethoxyphenethylamine	7.8	4-iodo-2,5-dimethoxyphenethylamine	79	6.1		
9	cocaine	3.4	cocaine	72	3.7		
	benzoylecgonine (minor component)	-5.3	NR ^c				
	ecgonine methyl ester (minor component)	-10.0	NR				
10	5-methoxy- <i>N,N</i> -di-isopropyltryptamine	6.5	5-methoxy- <i>N,N</i> -di-isopropyltryptamine	2.2	5.3		
11	heroin	7.6	heroin	62	5.9		
	6-monoacetylmorphine (MAM) (minor component)	-3.7	6-monoacetylmorphine (MAM) (minor component)				
12	5-methoxy- α -phamethyltryptamine	-3.8	5-methoxy- α -phamethyltryptamine	0.4	19		
13	<i>N,N</i> -dipropyltryptamine	5.5	<i>N,N</i> -dipropyltryptamine	74	4.6		
14	2,5-dimethoxyphenethylamine	-1.0	2,5-dimethoxyphenethylamine	53	7.0		
	2,4,5-trimethoxyphenethylamine (minor component)	1.2	NR				
15	MDMA (ecstasy)	5.9	MDMA (ecstasy)	19	0.76		
16	4-hydroxy- <i>N,N</i> -di-isopropyltryptamine/	-3.2	4-hydroxy- <i>N,N</i> -di-isopropyltryptamine/	59	7.2		
	4-hydroxy- <i>N,N</i> -dipropyltryptamine	/	NR	/			
	5,6-dimethoxy- <i>N</i> -isopropyl- <i>N</i> -methyltryptamine (minor component)	-3.2		59			
		-3.0	NR				
17	amphetamine	-9.2	amphetamine	38	0.34	41	-7.3
18	amphetamine	-8.7	amphetamine	27	4.5	25	8.0
19	amphetamine	-15.6	amphetamine	11	7.5	14	-21
20	amphetamine	-11.3	amphetamine	33	4.1	28	18
21	amphetamine	-12.9	amphetamine	17	7.4	18	-5.6

^a Mixture of two samples

^b Not included in LC-TOFMS mass library

^c NR, not reported

Identification of the active compounds in the street drug samples was straightforward since the number of drug-like components in the samples varied only from one to five, and the sample matrix was relatively simple compared to the biological material. An example TIC is shown in Figure 10. The LC-TOFMS results report listed only one entry per detected mass, except for sample 16, in which two entries of identical molecular formula were listed. Based on the accurate mass of the protonated molecule, identical molecular formulae cannot be differentiated, and

additional techniques should be used for identification. In sample 9, the reference method was not able to identify benzoylecgonine and ecgonine methyl ester, but these entries were obviously true positive findings as by-products of cocaine. The additional compounds found by the reference methods, such as desethyl chloroquine (sample 1), acetylcodeine (samples 1 and 11), piracetam (sample 4) and cinnamoylcocaine (sample 4), were not included in the LC-TOFMS database. In sample 14, a trace of 2,4,5-trimethoxyphenethylamine was detected by LC-TOFMS

and interpreted as a true positive finding, since the compound was structurally related to the main finding of 2,5-dimethoxyphenethylamine. In sample 16, the small peak of 5,6-dimethoxy-*N*-isopropyl-*N*-methyltryptamine was considered a false positive finding. The mean and median of the absolute values of the mass errors between calculated and measured mass were 7.2 and 5.9 ppm, respectively, but for one low-molecular-weight amphetamine, the mass error ranged from 3.2 to even 24 ppm.

The identified components were quantified using the single-calibrant LC-CLND method. Three parallel analyses in three dilutions were performed, and the concentration was calculated from the dilution, in which the peak area was within the range of the caffeine calibration standards. The amount of nitrogen per injected sample component was calculated directly from

the caffeine response curve, and the drug concentrations were calculated using the ratio of nitrogen content and molecular mass of the compound. An example LC-CLND chromatogram of a seized sample is shown in Figure 11. Quantitative results by reference methods were available for 11 sample components of the 32 identified compounds. The mean relative difference between the results by LC-CLND and the reference methods was 11%, and the range was 4.2-21%. A mean RSD of 6% was obtained for three parallel LC-CLND analyses. In sample 5, the amount of 5-methoxy-*N,N*-dimethyltryptamine was as high as 120%, possibly because of co-elution with a matrix component. This co-elution was not detected by LC-TOFMS, but the UV chromatogram indicated a fronting peak with a UV spectrum identical to the main peak.

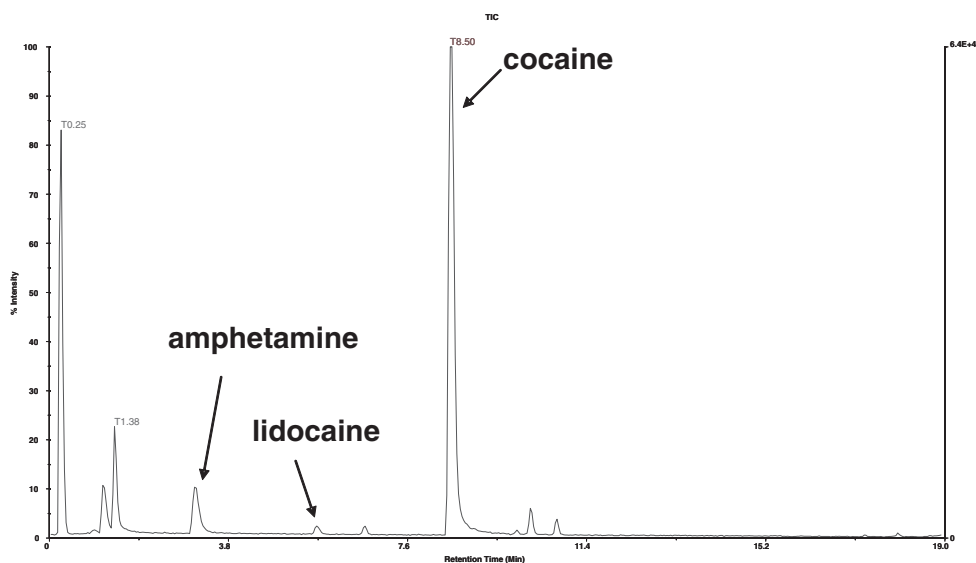


Figure 10. LC-TOFMS total ion chromatogram of a seized street drug sample, obtained after a simple dilution

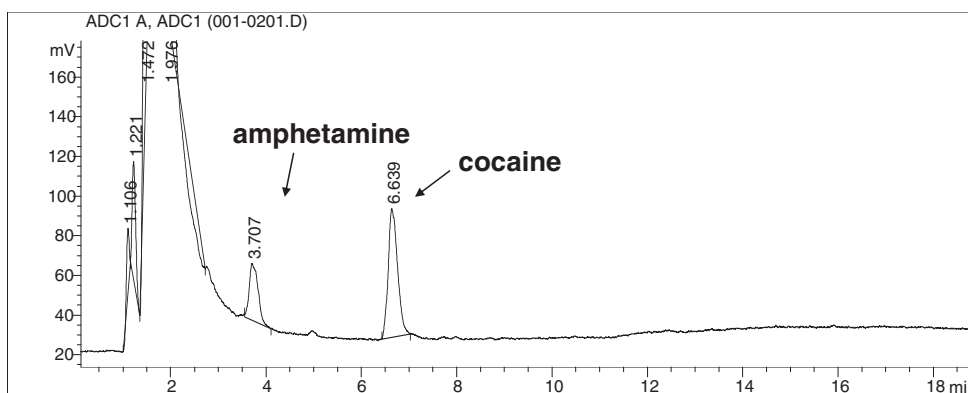


Figure 11. LC-CLND chromatogram for the sample of Figure 10, obtained after a simple dilution

The present approach combining LC-TOFMS and LC-CLND was an extremely quick and straightforward way of analysing drugs in seized material. The sample compounds were identified properly by accurate mass measurement, provided that the corresponding entries were included in the database. The same precautions concerning identification apply here as mentioned in the previous studies (I, II). LC-CLND is an effectual tool for the quantification of substances in powdered samples, which generally do not require advance extraction and purification. Criminalistics laboratories would evidently increase their throughput by using fewer and more efficient methods, such as the present two, instead of applying a range of dedicated target methods.

5.3. Quantification of drugs in blood by LC-CLND

5.3.1. Basic lipophilic drugs (IV)

Quantification of basic lipophilic drugs in blood specimens was studied by LC-CLND, based on the detector's equimolar response to nitrogen, and by using caffeine as a single secondary standard. Because of the complexity of the biological matrix, an extraction procedure was ap-

plied prior to chromatography. Analysis without PRS necessitated the estimation of the mean recovery of extraction for basic lipophilic drugs in plasma and whole blood by using representative model compounds. The validity of the established mean extraction recoveries was verified by analysing six proficiency test samples by the single-calibrant LC-CLND method.

LLE recoveries with butyl chloride-isopropyl alcohol for basic lipophilic drugs were determined from blood specimens spiked with the respective reference standards. The study was carried out with 33 drugs, representing antidepressants, antihistamines, antipsychotics, cardiovascular drugs and opioids (Table 3). The drug was considered basic and lipophilic if the compound's calculated log D value (octanol/water) at the extraction pH of 11 was greater than 1.5 and an aliphatic amino group was present in the molecule. Amphoteric drugs were not analysed in the study, except for pentazocine, which, despite containing a phenolic hydroxyl group, is sufficiently lipophilic to be extracted outside of the optimal pH. The recoveries were determined at two concentrations, 0.2 and 1.0 mg/l, representing therapeutic and toxic levels, respectively. Particularly low dose-drugs were not included in the study. The recoveries were determined by LC-CLND in four parallel analyses spiked with the PRS in a regular manner. The mean extraction recoveries in plasma at 0.2 mg/l and 1.0 mg/

I levels were 88 ± 16 and $92 \pm 16\%$, respectively, and in whole blood 80 ± 17 and $87 \pm 16\%$, respectively. The grand mean extraction recoveries based on concentrations in both plasma and

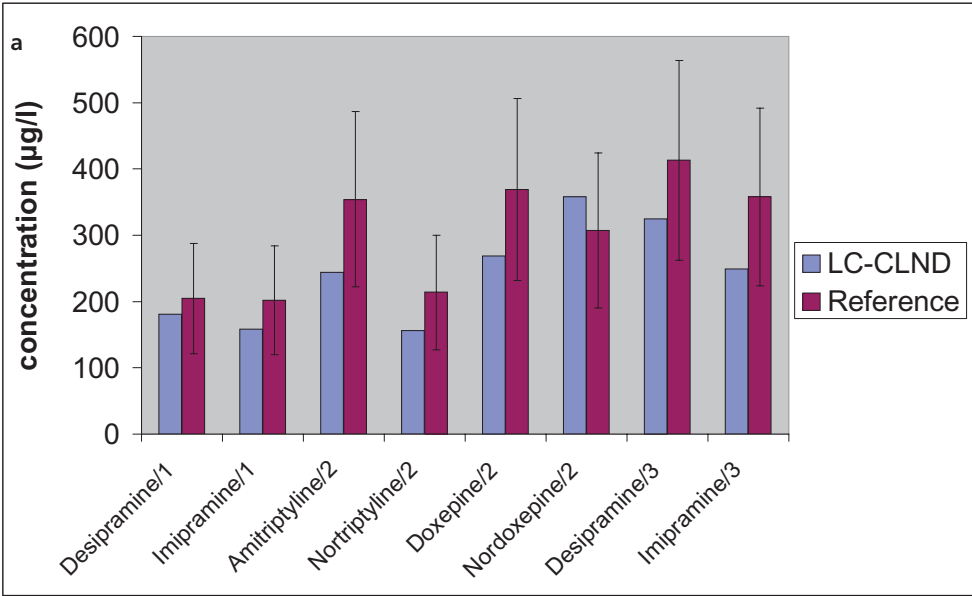
whole blood were 90 ± 18 and $84 \pm 16\%$, respectively. The RSD of four parallel injections was below 15%.

Table 3. Liquid-liquid extraction (LLE) recoveries with butyl chloride-isopropyl alcohol for basic lipophilic drugs from blood specimens.

Compound	Log D	Recovery of extraction							
		Plasma				Whole blood			
		0.2 mg/l	RSD (%)	1.0 mg/l	RSD (%)	0.2 mg/l	RSD (%)	1.0 mg/l	RSD (%)
Amitriptyline	4.91	78	3	79	8	84	5	66	9
Aripiprazol	5.59	87	4	100	3	68	6	61	14
Bisoprolol	2.13	-	-	-	-	89	11	95	2
Chlorpromazine	5.19	51	12	107	2	103	5	98	2
Citalopram	2.50	102	7	87	11	97	4	85	4
Clomipramine	5.51	96	2	95	3	62	4	94	7
Clozapine	3.48	96	4	80	5	85	4	72	10
Desipramine	4.04	98	6	95	5	75	10	81	8
Dibenzepin	1.76	57	5	99	6	70	3	82	10
Diphenhydramine	3.66	98	3	97	2	68	4	98	1
Fluoxetine	4.04	64	9	118	5	71	2	86	14
Fluvoxamine	3.10	108	3	58	10	116	11	111	6
Imipramine	4.79	90	2	116	1	82	4	99	10
Levomopromazine	4.93	89	3	96	3	42	10	38	14
Methadone	4.19	99	2	98	3	88	9	86	11
Metoprolol	1.78	86	14	69	8	88	9	98	2
Mianserine	3.67	68	1	85	9	72	11	61	10
Mirtazapine	2.75	-	-	-	-	68	8	72	9
Norcitalopram	3.13	92	6	98	2	105	5	112	3
Norclomipramine	4.77	97	5	96	5	74	10	88	9
Normethadone	2.76	88	2	101	2	97	2	95	7
Nortramadol	1.88	98	2	91	3	99	3	102	1
Nortrimipramine	4.38	99	5	102	4	72	9	99	3
Nortriptyline	5.61	109	5	82	5	89	4	92	7
Pentazocine	3.58	-	-	-	-	76	4	68	9
Promazine	4.62	66	5	72	7	47	5	78	7
Propranolol	3.09	96	4	93	6	98	9	98	8
Quetiapine	1.57	96	2	97	4	70	12	104	4
Thioridazine	6.11	57	9	47	11	63	6	84	11
Tramadol	2.49	101	5	94	8	93	9	109	2
Trimipramine	5.14	85	3	121	5	68	4	73	17
Venlafaxine	2.90	-	-	-	-	95	5	94	1
Verapamil	3.89	110	3	99	3	65	5	93	4
Mean		88	5	92	5	80	6	87	7
Median		94	4	96	5	76	5	92	7

The validity of the single-calibrant LC-CLND method was studied by analysing proficiency test samples, without PRS, using the universal extraction recoveries established (90% for plasma and 84% for whole blood) (Figure 12a and 12b). Based on eight determinations in plasma and 12 in whole blood samples, the mean accuracy in plasma and whole blood was 24 and 17%, respectively. The maximum error was 31% for both specimens. Repeatability was studied by performing four parallel analyses: the RSD for

plasma and whole blood was 19 and 17%, respectively. The standard score (z-score) indicates how many standard deviations an observation is above or below the mean, allowing comparison of observations from different normal distributions. Based on the proficiency test results, the z-score of the single-calibrant LC-CLND method was better than one (excellent) in plasma for two out of eight substances and in whole blood for nine out of 12 substances. The z-score was better than two (good) for all results.



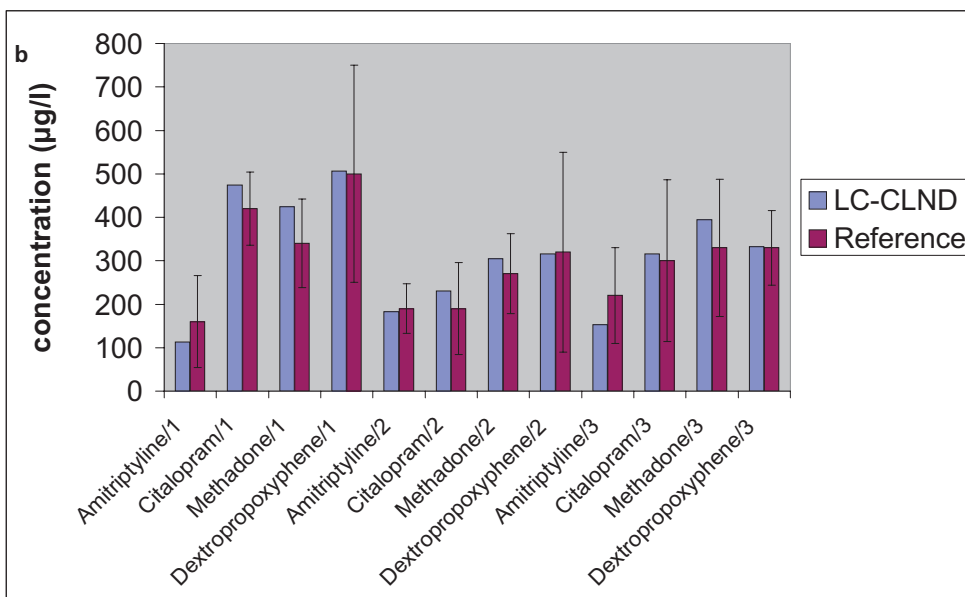


Figure 12a ja 12b. LC-CLND results obtained without primary reference standards for plasma (a) and whole blood (b) proficiency test samples. Error bars for the reference values represent 95% confidence limits.

The butyl chloride-isopropyl alcohol extraction method yielded relatively clean extracts, resulting in low background noise in the LC-CLND chromatograms (Figure 13). Butyl chloride was chosen as an extraction solvent based on its proven applicability to basic lipophilic drugs in analytical toxicology. Recognised already in the 1970s (Siek 1978), butyl chloride has been found to provide clean and efficient extraction with a list of recoveries from an aqueous buffer at pH 9 (Demme 2003) reported for over 200 toxicologically relevant substances. Isopropyl alcohol was

added to the extraction solvent to improve the recovery of polar compounds. Emulsion formation was observed in individual cases, especially with plasma samples, and the phenomenon was found to significantly affect the recoveries of the late-eluting, mostly lipophilic compounds. In cases where visible emulsion formation was observed, the sample preparation was repeated. For reducing emulsion formation, samples were extracted in a rotary shaker instead of a vortex mixer.

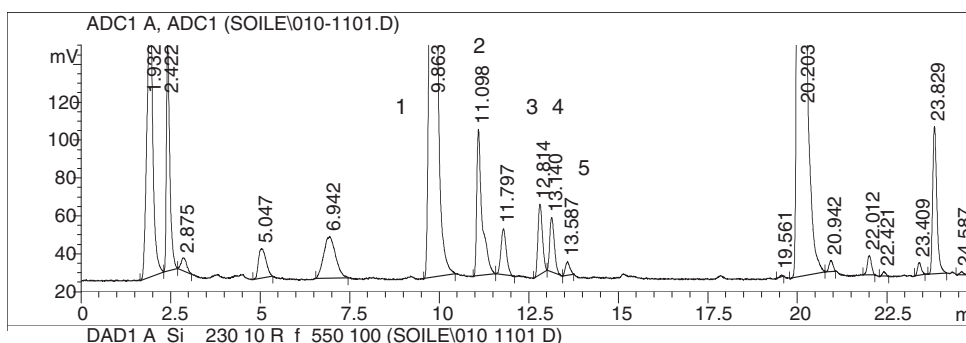


Figure 13. LC-CLND chromatogram for a whole blood proficiency test sample after liquid-liquid extraction, showing caffeine (1), citalopram (2), dextropropoxyphene (3), methadone (4) and amitriptyline (5).

Efficient LC separation plays a key role in LC-CLND analysis. The chromatographic separation was developed for basic lipophilic compounds, without optimising the resolution for any particular set of compounds. As the mobile phase must be volatile and free of nitrogen, the repertoire of suitable buffers was limited, and methanol was used instead of acetonitrile. Overall, the study was limited to drugs for which no co-eluting compounds from the matrix were detected, and hence clomipramine and norclomipramine, for example, were excluded from the plasma analyses.

The present study was the first to apply LLE in a single-calibrant LC-CLND analysis. The repeatability of the LLE method was found to be sufficiently constant for forensic and clinical toxicology purposes. In the methods published earlier, protein precipitation has been used in sample preparation (Deng et al. 2004). However, during the development of the present method, both protein precipitation and the SPE approach produced unsatisfactory results, even when using automatic sample preparation. LC-CLND functioned well with LLE-treated biological samples, and even after thousands of injections, no major maintenance operations were needed.

5.3.2. *Tramadol and metabolites (V)*

The opioid analgesic drug tramadol was taken as an example of the use of the single-calibrant LC-CLND method in a clinical context. After a single dose of 100 mg tramadol to four volunteers, tramadol and its two main metabolites, *O*-desmethyltramadol and nortramadol, were quantified from plasma samples without PRS by LC-CLND, applying the extraction recoveries obtained with model compounds. The study was applied to a pharmacogenetic setting by deter-

mining the tramadol metabolite ratios and the *CYP2D6* genotypes for each study subject.

The extraction recoveries were determined for the three analytes (tramadol, *O*-desmethyltramadol, and nortramadol) for the two model compounds for extraction (venlafaxine and ketobemidone) and for the internal control (dextropropoxyphene) in four parallel analyses of plasma spiked at concentrations of 0.05, 0.15 and 0.50 mg/l. The structures of the compounds and their log *D* values (octanol/water) at different pH values are shown in Figure 14a and b. The model compounds with chemical properties similar to tramadol and its metabolites were used for establishing the extraction recoveries from plasma. Venlafaxine, as a basic compound possessing a tertiary aliphatic amine group, represented tramadol and nortramadol. Ketobemidone with a tertiary aliphatic amine group and a phenolic hydroxyl group is amphoteric and represented *O*-desmethyltramadol. Dextropropoxyphene was used as an internal control to monitor the quality of sample preparation and chromatography; however, it was not used to correct the results. The standard deviation (SD) of day-to-day retention times over a three-week period was below 0.03 min. High caffeine concentrations were found to interfere with nortramadol under the present chromatographic conditions, and consequently the study subjects were advised to refrain from consuming caffeine for two days before sampling. The LOQ for tramadol, nortramadol and *O*-desmethyltramadol was 15, 15 and 30 µg/l, respectively. These limits were sufficiently low for analysing the compounds at therapeutic concentrations, although the required plasma sample volume was as high as 5 ml. The LOQs obtained were at the same level as those reported for GC-MS (Goeringer et al. 1997), but not as low as with LC coupled to fluorescence detection (Rouini et al. 2006).

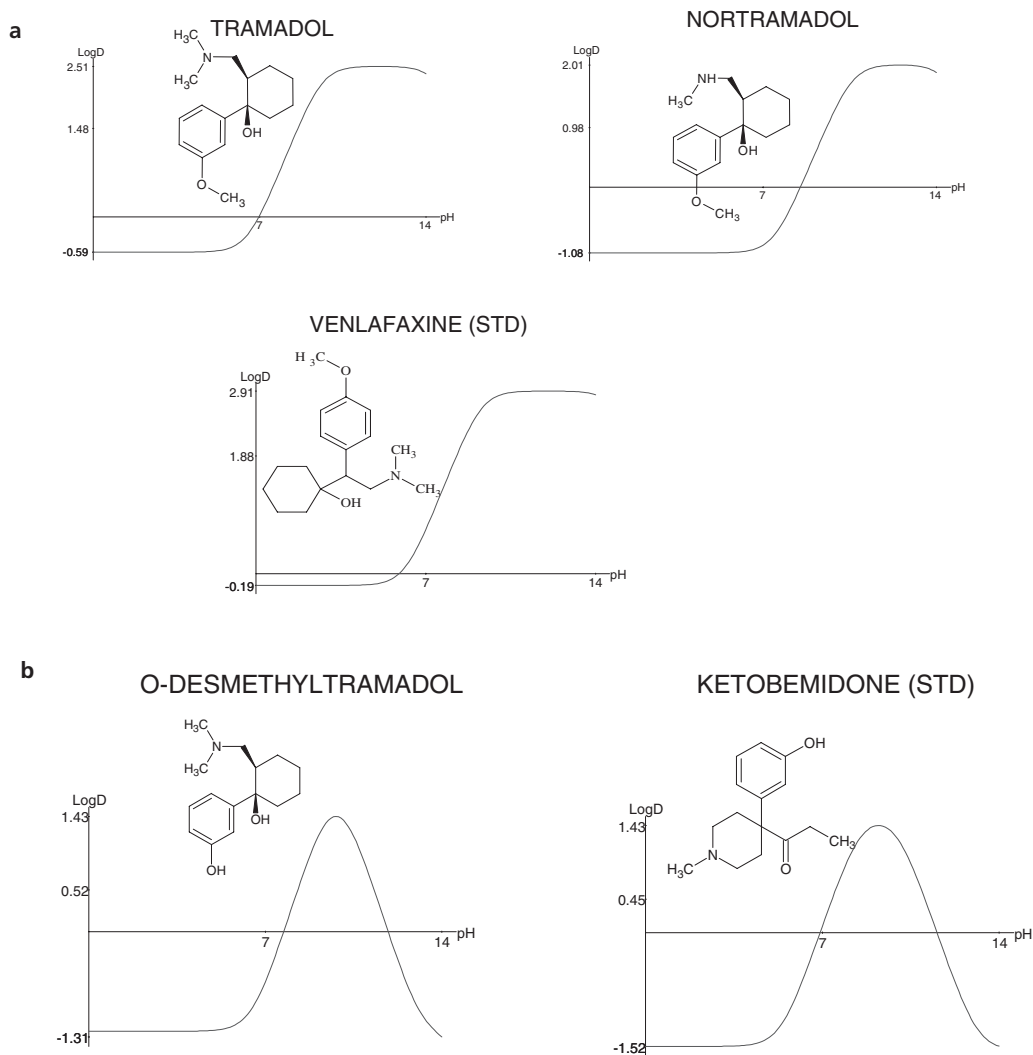


Figure 14. Structures and predicted log D values at different pH using ACDLabs 8.0 software: Basic analytes, tramadol and nortramadol, and their model compound venlafaxine (a); Amphoteric analyte *O*-desmethyltramadol and its model compound ketobemidone (b).

The mean extraction recoveries for tramadol, nortramadol and *O*-desmethyltramadol were 88%, 95% and 32%, respectively, with an RSD between 11 and 14%. The mean extraction recoveries for the model compounds, venlafaxine and ketobemidone, were 89% and 30%, respectively. The single-calibrant LC-CLND analysis of the study subjects' plasma samples included the following procedure: the recovery of venlafaxine (89%) was used for correcting tramadol

and nortramadol analysis, and the recovery of ketobemidone (30%) was used for correcting *O*-desmethyltramadol analysis. The LC-CLND results were compared with those obtained by a GC-MS reference method (Figure 15). The mean difference between the results of the two methods for tramadol, nortramadol and *O*-desmethyltramadol was 8%, 32% and 19%, respectively, and the range was 0-60%. These values represent typical method-to-method differences, and

the mean values are similar to or only slightly higher than the bioanalytical within-method validation criteria of 15-20% for accuracy and precision (Shah et al. 2000b). Despite of the relatively high differences between LC-CLND and GC-MS results, the magnitude of the results was similar. The cytochrome P450 (CYP) isoenzyme CYP2D6 has been shown to convert tramadol to *O*-desmethyltramadol, while CYP3A4 is believed to convert tramadol to nortramadol (Paar 1992). CYP2D6 is generally polymorphic, and it has implications for tramadol/*O*-desmethyltramadol ratios. Figure 15 shows the number of functional *CYP2D6* genes of each of the four study subjects. Subject 1 had an ultrarapid metaboliser phenotype with active whole gene duplication, and subjects 2-4 had an extensive metaboliser

phenotype with one or two functional genes. A correlation between the number of functional genes and the tramadol/*O*-desmethyltramadol plasma metabolite ratios was found in the study, despite the small subject pool. The metabolic ratios obtained for extensive metabolisers were, irrespective of technique, comparable to the range reported earlier (Borlak et al. 2003). The present study shows that single-calibrant LC-CLND can be used to provide metabolic ratios of tramadol and other substances within therapeutic drug monitoring and toxicology, if model compounds are used to establish the extraction recoveries. A disadvantage with biological samples is the limited sensitivity of the detector, for which a 5 ml sample volume was required.

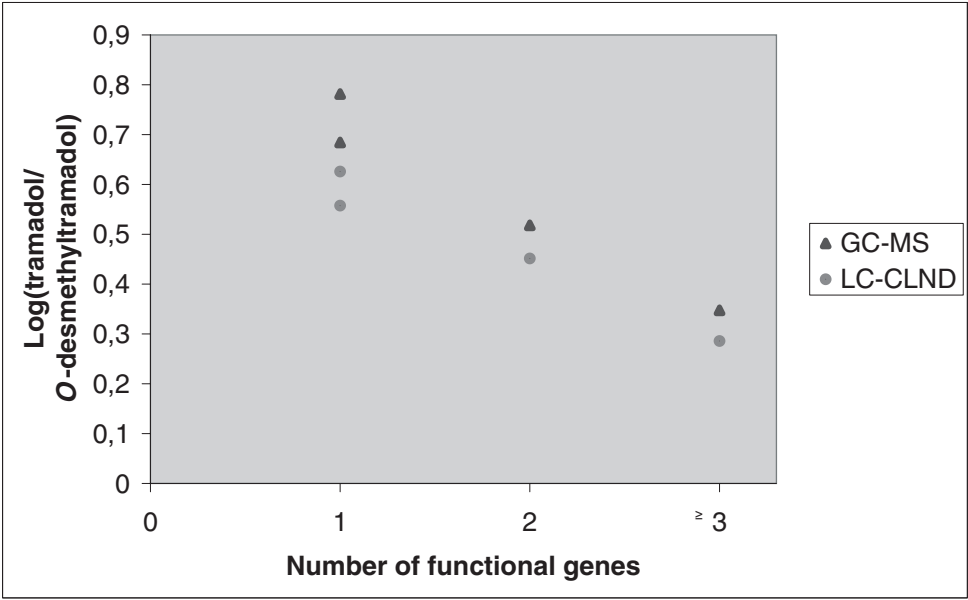


Figure 15. The number of functional *CYP2D6* genes and the corresponding metabolite ratios measured by LC-CLND and GC-MS.

6. GENERAL DISCUSSION

The following three dimensions can be associated with the quality of the outcome of forensic and clinical drug analysis: 1) qualitative analysis should cover a wide spectrum of toxic and abused substances with a high degree of reliability, 2) quantitative analysis should measure the quantity of the relevant substances with reasonable accuracy, e.g. differentiating therapeutic, toxic and lethal concentrations, and 3) the analysis should be completed within an acceptable period of time. Unequivocal determination of the identity of compounds is of crucial importance in forensic science and toxicology because of legal consequences, and failure in analysis may lead to overturning of court convictions and a loss of confidence in the laboratory (Hibbert 2003). In a clinical context, incorrect analysis may result in a misdiagnosis and costly unnecessary treatment, or worse, in the lack of necessary treatment.

There is no general agreement on how a sufficient level of certainty should be achieved in the qualitative bioanalysis of drugs. In his polemical review, de Zeeuw concluded that substance identification is a neglected and misunderstood domain in analytical toxicology and suggested rapid and concerted actions to improve general knowledge, to define uniform strategies in the analytical approach and in the interpretation of results, and to set up and maintain suitable banks of reference substances and computerised databases to allow unambiguous identification (de Zeeuw 2004). The information power of different analytical techniques has, in fact, been discussed (Hartstra et al. 2000) using several mathematical approaches, such as mean list length method, discriminating power (Mof-fat et al. 1978) and information content (Mas-sart 1973). But these theoretical considerations, though valuable in themselves, give little practical advice for judging the validity of a particular result in a particular case. An extensively cited review by Rivier (Rivier 2003) is more practically oriented and brings together detailed guidelines and requirements for chromatographic and spectrometric techniques from different organi-

sations, including WADA, the International Association of Forensic Toxicologists, the Centre for Veterinary Medicine of the US Food and Drug Administration, and the European Union (EU). Very detailed rules are presented in EU Commission Decision 2002/657/EC implementing Council Directive 96/23/EC, concerning performance of analytical methods and the interpretation of results for laboratories involved in animal and meat residues analyses (European Union 2002). For instance, MS techniques measuring specific ions were valued according to the number of information points (IP) related to the technique. Interestingly, LC-MS was given an IP of 1.0 for an ion (or a precursor ion) and 1.5 for a product ion, but for high resolution LC-MS, these values were 2.0 and 2.5, respectively. The RRT criteria for LC were <2.5%. To attain the minimum requirement of 4.0 IPs, at least two low-resolution MRM ion transitions are required or one at high resolution. A proposal to specify and change these rules has been recently published, stating that at least three TOFMS ions would be required versus four low-resolution MS ions in order to achieve the minimum of 4.0 IPs (Nielen et al. 2007). However, these considerations cannot be directly extended to analysis without PRS, in which the chromatographic identification parameter is missing.

The LC-TOFMS methods presented in this thesis were the first to involve accurate mass-based identification against a comprehensive database in a high-throughput manner in biological samples. Although some LC-TOFMS screening methods have been published earlier (Zhang et al. 2000a, Maizels and Budde 2001, Nielen et al. 2001), those methods were limited to a low number of substances and lacked the data processing properties necessary for high-throughput analysis. The success of the present methods was based on the unique approach of a post-run reverse target database search (Gergov et al. 2001a), optimised extraction and chromatography, and the improved properties of the modern orthogonal acceleration TOFMS analysis-

ers, featuring accurate mass analysis over a wide dynamic range. From the vast amount of full-spectrum data gathered during an LC-TOFMS run, relevant information can be retrieved immediately, or afterwards if a new question is posed. Hence, although clearly being target screenings, the present methods possess similar potential for systematic toxicological analysis as those that involve the IDA approach (Decaestecker et al. 2000). When LC-TOFMS analysis is based on accurate molecular mass, isotopic pattern, metabolite pattern and chromatographic RT, very reliable identification is obtained, fulfilling the requirements of confirmation analysis. However, if the appropriate PRS is not available, the result should be considered tentative and confirmed by another independent technique. The current developments in QTOF-MS technology obviously allow an analogous screening procedure to be developed, but include an additional confidence level for identification of unknown compounds based on CID and fragmentation prediction (Sweeney 2007, Hill et al. 2008, Stranz et al. 2008).

Some scientists think that the chances of finding the identity of an unknown compound increase with the total number of reference spectra in the database (Aebi and Bernhard 2002). Thurman et al. proposed a scheme for using LC-TOFMS with a very large database search for pesticide residues in tomato skins (Thurman et al. 2005). The method first involved initial detection of a possible unknown pesticide in actual marketplace vegetable extracts by using accurate mass and by generating empirical formulae, then searching either the Merck Index database on CD (10,000 compounds) or the ChemIndex (77,000 compounds) for possible structures. Subsequently, an ion-trap MS instrument was required to measure MS/MS spectra, followed by fragment ion identification using chemical drawing software and comparison with accurate-mass ion fragments. Finally, verification was performed with authentic standards, if available. Based on the three examples provided, the approach is innovative, but rather laborious.

Polettini et al. further extended the approach of LC-TOFMS screening with very large databas-

es. They used capillary electrophoresis (CE) for separation, and utilised a subset of the large PubChem Compound database (National Institutes of Health, USA) as a reference database (Polettini et al. 2008). This database contains approximately 50,500 compounds, including biologically active small molecules, pharmaceutical and illicit drugs, pesticides and poisons. The average number of hits with identical chemical formula was 1.82 ± 2.27 , with a median of one and range from one to 39. However, the probability of a search retrieving different entries with identical chemical formula was higher than with smaller databases. The authors acknowledged that additional information, such as history or circumstantial data, concomitant presence of parent drug and metabolite, selective sample preparation, liquid chromatographic retention, and CE migration behavior, must be used in order to tighten the focus of the search.

Kind and Fiehn studied accurate mass measurement and isotopic pattern in the field of metabolomics (Kind and Fiehn 2006). Metabolomics tries to identify and quantify all metabolites in a given biological context. Generating more than 1.6 million molecular formulae in a range of 0-500 Da while strictly observing mathematical and chemical rules, they concluded that a mass spectrometer capable of 3 ppm mass accuracy and 2% error for isotopic abundance patterns outperforms mass spectrometers that have less than 1 ppm mass accuracy as well as hypothetical mass spectrometers with 0.1 ppm mass accuracy that do not include isotope information in the calculation of molecular formulae (Kind and Fiehn 2006). This finding, though based on a different analytical context, supports a greater significance for isotopic pattern determination, as was also found in the present thesis.

In forensic and clinical drug analysis, the use of accurate mass measurement with very large databases may be feasible in instances where much effort and time have to be put into solving a single important case. However, high-throughput screening that focuses on the prevalent drugs and poisons, as described in this thesis, has shown its applicability in practice. In Finland, roughly 80% of fatal poisonings are due to only

20 different drugs. The use of very large databases necessarily results in a number of false positive findings that require extra resources in terms of interpretation and time-consuming confirmation analyses.

Despite the immense value of reliable broad-spectrum identification, many application areas are essentially dependent on quantitative results, threshold values or cut-off limits (Wennig 2000). The performance of single-calibrant LC-CLND has been extensively studied in terms of linear range, accuracy and reproducibility. The linear range for diverse compounds via flow injection ranged from 0.05 to 5 mM nitrogen, and the absolute response exhibited an average error of <10% among the compounds (Shah et al. 2000a). In another study, the response was found to be close to quantitative, depending on structures, with a variation of 10-20% when compounds contained isolated nitrogen atoms (Yan et al. 2007). Regarding compounds with adjacent nitrogen atoms connected by a single bond, e.g. triazoles or pyrazoles, the CLND response was highly structure-dependent. In these cases, a structurally similar calibration compound should be used in quantification. The day-to-day reproducibility of calibration curves remained constant at least for 15 days (Bhattachar et al. 2006), and the long-term reproducibility based on indole calibrant peak area proved to be approximately $\pm 10\%$ (Lane et al. 2005). Another study indicated that the caffeine calibration curve was stable for longer than one week of continuous use, but the use of a control sample of caffeine within a sample set was recommended (Corens et al. 2004). These findings suggest that LC-CLND analysis is feasible in the combinatorial chemistry environment. The studies of the present thesis widened the scope of LC-CLND to biological material, showing the robustness of the method with more difficult samples. Here, calibration was performed at the beginning of each run, and the standards were included again at the end of each sample set for controlling the stability of calibration.

Nitrogen specificity limits the use of mobile phases in LC-CLND analysis. Reversed phase LC at acidic pH has been commonly used in separa-

tion because of the lack of volatile, nitrogen-free bases. The organic phase was generally methanol or isopropyl alcohol since acetonitrile cannot be used with LC-CLND. However, when using isopropyl alcohol, the back-pressure can rapidly increase too high, particularly when small particle size LC columns are involved. Trifluoroacetic acid has been used as an organic modifier in the LC for improving the separation of carboxylic acids, and it was also found to provide more efficient mass transfer of basic compounds with amine groups, resulting in reduced peak tailing (Chan and Fujinari 2004). Mobile phase flow rate in LC-CLND should be less than 0.4 ml/min (Nusbaum et al. 2002). Because of the limitations described above, the optimisation of LC separation is challenging. In the present thesis, chromatographic optimisation software, particularly DryLab, were used to facilitate LC method development for separating basic lipophilic drugs. However, the separation is always a compromise between the run time, sensitivity and resolution.

Sample preparation for LC-CLND bioanalysis is a demanding task. A complex biological matrix does not allow samples to be injected directly into the system, and all sample preparation procedures necessarily affect the recovery of drugs. Protein precipitation with methanol has been used in an LC-CLND analysis of plasma and urine samples (Deng et al. 2004). In the present thesis as well, protein precipitation was investigated during the method development stage, but the analytical background from the matrix was too high and prevented sensitive detection. SPE has been applied only in a single report, for the determination of imidacloprid in fruits and vegetables (Ting et al. 2004). The extraction recovery for various sample materials was relatively constant at 90% with an RSD of only 8%, but CLND was used solely as a nitrogen-selective detector. In bioanalytical method validation, extraction recovery has not been among the validation parameters regarded as essential as long as the data for LOQ or LOD, precision and accuracy (bias) are acceptable (Peters et al. 2007). This is due to the fact that an internal standard, preferably deuterium-labelled, compensates for the changes in recovery. Single-calibrant LC-CLND applied

to bioanalysis sets new challenges for developing robust extraction methods with high precision, as well as for the prediction of extraction recoveries based on the analyte structure.

The time factor – the third dimension of quality – presumes that at least the preliminary results of an investigation are completed within a reasonable period of time, which varies from a few hours in clinical toxicology to a few weeks in forensic science. It is well understood that rare research chemicals for basic research must often be solicited from a colleague scientist, but this should not be the case with official forensic and clinical work. While the search for an organisation willing to provide professional laboratories with a rapid access to PRS of drugs is underway, the laboratories are obliged to develop analysis methods that are less dependent on the reference standards and capable of producing timely analytic results with reasonable certainty. The present study has introduced the performance of one such method involving the combined use of LC-TOFMS and LC-CLND.

Combining LC-TOFMS and LC-CLND by splitting the mobile phase flow into both detectors

for simultaneous identification and quantification may be an obvious idea. This has indeed been realised in combinatorial chemistry (Yurek et al. 2002), where the amount of sample and the concentration of analytes are usually sufficiently high. In forensic science, too, the combination of these instruments appears promising for analysing seized drugs and even impurity profiles. However, the sensitivity of CLND is considerably lower than that of TOFMS, and this may cause incompatibility problems in bioanalysis, as shown earlier (Deng et al. 2004). In addition, CLND is perhaps too complex to be used solely as a nitrogen-selective detector without utilizing its *N*-equimolar response. A feasible approach in bioanalysis would involve quantitative analysis by LC coupled with diode-array UV detection (Pragst et al. 2004), exploiting the stable calibration properties of the technique, and adding CLND to the system in cases where PRS are not available. This would allow, for example, the determination of important metabolites together with their parent drugs, which would significantly facilitate the interpretation of forensic and clinical cases.

7. CONCLUSIONS

Urine drug screening for drugs by positive ion LC-TOFMS, based on a large target database of exact monoisotopic masses, metabolic patterns, and LC retention times, if available, proved to be feasible in forensic toxicological practice. The isotopic pattern (SigmaFit) was taken into use for the first time as part of a routine MS database search, and matching of the theoretical calculated isotopic pattern against the measured pattern further improved, revealing the correct molecular composition from a mass spectrum. The automated acquisition of correct SigmaFit values and accurate masses were proven over a wide dynamic range, the mean mass error being 2-3 ppm. The data obtained in this study justify the use of the limit values of 0.03 and 10 ppm for SigmaFit and mass tolerance, respectively. The present approach makes tentative identification possible in urine drug screening without immediate need for PRS. Additional proof can be obtained from the interpretation of MS spectra after CID experiments (I, II).

Qualitative analysis by LC-TOFMS followed by quantitative analysis by single-calibrant LC-CLND allowed the instant characterisation of seized material for both scheduled and design-

er drugs without PRS, with practically unlimited potential for updating the target database with new compounds. Possessing equimolar response to nitrogen, LC-CLND allows quantitative analysis of drugs without access to PRS.

The mean relative difference between results of LC-CLND and the reference methods was only 11%, suggesting that the accuracy of quantification is appropriate for use in forensic science (III).

Single-calibrant LC-CLND analysis proved to be feasible for basic lipophilic drugs in plasma and whole blood samples in a toxicological context following establishment of the mean extraction recovery. The results obtained by LC-CLND without PRS deviated on average 20% from the certified reference values of proficiency test samples. As exemplified by the analysis of tramadol and its main metabolites, LC-CLND was capable of producing clinically relevant concentration data down to therapeutic levels in 5 ml plasma samples. Further attention should be paid to developing generic extraction methods with steady recovery and utilisation of model compounds for recovery prediction (IV, V).

8. ACKNOWLEDGEMENTS

This work was carried out at the Laboratory of Toxicology, Department of Forensic Medicine, University of Helsinki, during 2003-2007. I wish to thank Professor Erkki Vuori, Head of the Department of Forensic Medicine, for his encouraging attitude towards me and for providing superb working facilities in a top-quality laboratory. Financial support from Helsinki University Research Funds is also gratefully acknowledged.

I express my deepest gratitude to my main supervisor, Docent Ilkka Ojanperä, for the primary ideas of the thesis and for orienting me to scientific thinking. His support and advice in written reporting has been invaluable. I warmly thank my second supervisor, Dr. Anna Pelander, especially for her guidance and instructions concerning the technical performance of analytical instrumentation.

I sincerely thank the two reviewers of this thesis, Docent Pirjo Lillsunde and Docent Pertti Koivisto, for their constructive comments on the manuscript.

I am grateful to Soile Tuominen for her excellent technical assistance in LC-CLND experiments, and to Dr. Ilpo Rasanen and Dr. Johanna Sistonen for their collaboration in GC-MS and pharmacogenetic experiments, respectively. I also thank Elisa Ali-Tolppa, Dr. Ilmari Krebs, Dr. Mathias Pelzing, Dr. Erkki Sippola and all of the personnel of the Laboratory of Forensic Toxicology.

All friends and relatives are thanked for empathy and emotional support.

My warmest thanks for everything go to my family, Ilkka and Aino, and to the expected baby for giving an impetus to finally complete this thesis during my nursing leave.

Helsinki, November 2008

Suvi Ojanperä

9. REFERENCES

- Aalberg L, Andersson K, Bertler C, Boren H, Cole MD, Dahlen J, Fannon Y, Huizer H, Jalava K, Kaa E, Lock E, Lopes A, Poortman van der Meer A, Sippola E.** Development of a harmonised method for the profiling of amphetamines - I. Synthesis of standards and compilation of analytical data. *Forensic Sci Int* 2005;149:219-29.
- Aebi B, Bernhard W.** Advances in the use of mass spectral libraries for forensic toxicology. *J Anal Toxicol* 2002;26:149-56.
- Amster IJ.** Fourier transform mass spectrometry. *J Mass Spectrom* 1996;31:1325-37.
- Balogh MP.** Debating resolution and mass accuracy. *LC-GC Europe* 2004;17:152-9.
- Barantin L, Le Pape A, Akoka S.** A new method for absolute quantitation of MRS metabolites. *Magn Reson Med* 1997;38:179-82.
- Baselt RC.** *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed. Foster City: Biomedical Publications; 2004.
- Belov ME, Gorshkov MV, Udseth HR, Anderson GA, Smith RD.** Zeptomole-sensitivity electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry of proteins. *Anal Chem* 2000;72:2271-9.
- Beynon JH. 1954.** Qualitative analysis of organic compounds by mass spectrometry. *Nature* 1954;174:735-37.
- Bhattachar SN, Wesley JA, Seadeek C.** Evaluation of the chemiluminescent nitrogen detector for solubility determinations to support drug discovery. *J Pharm Biomed Anal* 2006;41:152-7.
- de Boer D, Bosman IJ, Hidvegi E, Manzoni C, Benko AA, dos Reis LJ, Maes RA.** Piperazine-like compounds: A new group of designer drugs-of-abuse on the European market. *Forensic Sci Int* 2001;121:47-56.
- Bogusz M, Erkens M.** Reversed-phase high-performance liquid chromatographic database of retention indices and UV spectra of toxicologically relevant substances and its interlaboratory use. *J Chromatogr A* 1994;674:97-126.
- Bogusz MJ, Maier RD, Krüger KD, Webb KS, Romeril J, Miller ML.** Poor reproducibility of in-source collisional atmospheric pressure ionization mass spectra of toxicologically relevant drugs. *J Chromatogr A* 1999;844:409-18.
- Borlak J, Hermann R, Erb K, Thum T.** A rapid and simple CYP2D6 genotyping assay - Case study with the analgetic tramadol. *Metabolism* 2003;52:1439-43.
- Brewer WE, Galipo RC, Sellers KW, Morgan SL.** Analysis of cocaine, benzoylecgonine, codeine, and morphine in hair by supercritical fluid extraction with carbon dioxide modified with methanol. *Anal Chem* 2001;73:2371-6.
- Bristow AW, Webb KS.** Intercomparison study on accurate mass measurement of small molecules in mass spectrometry. *J Am Soc Mass Spectrom* 2003;14:1086-98.
- Bristow AW, Webb KS, Lubben AT, Halket J.** Reproducible product-ion tandem mass spectra on various liquid chromatography/mass spectrometry instruments for the development of spectral libraries. *Rapid Commun Mass Spectrom* 2004;18:1447-54.

- Bristow AW.** Accurate mass measurement for the determination of elemental formula--a tutorial. *Mass Spectrom Rev* 2006;25:99-111.
- Bristow T, Constantine J, Harrison M, Ca-voit F.** Performance optimisation of a new-generation orthogonal-acceleration quadrupole-time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 2008;22:1213-22.
- Bruins AP.** Mass spectrometry with ion sources operating at atmospheric pressure. *Mass Spectrom Rev* 1991;10:53-77.
- Brunelli C, Gorecki T, Zhao Y, Sandra, P.** Corona-charged aerosol detection in supercritical fluid chromatography for pharmaceutical analysis. *Anal Chem* 2007;79: 2472-82.
- Burlingame AL.** Assessment of the trace organic molecular composition of industrial and municipal wastewater effluents by capillary gas chromatography/real-time high-resolution mass spectrometry: a preliminary report. *Ecotoxicol Environ Saf* 1977;1:111-50.
- Chan KM, Fujinari EM.** A reversed-phase HPLC method using non-nitrogen-containing mobile phases: Capacity factor, mobile phase, and column selectivity for amoxicillin, saccharin, and caffeine. *Am Lab* 2004;36:18-22.
- Chernushevich IV, Ens W, Standing KG.** In *Electrospray Ionization Mass Spectrometry*; Cole RB, Ed; New York: Wiley-Interscience, 1997. p. 203-34.
- Chernushevich IV, Loboda AV, Thomson BA.** An introduction to quadrupole-time-of-flight mass spectrometry. *J Mass Spectrom* 2001;36:849-65.
- Corens D, Carpentier M, Schroyen M, Meerpoel L.** Liquid chromatography-mass spectrometry with chemiluminescent nitrogen detection for on-line quantitative analysis of compound collections: Advantages and limitations. *J Chromatogr A* 2004;1056:67-75.
- Dams R, Huestis MA, Lambert WE, Murphy CM.** Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: Influence of ionization type, sample preparation, and biofluid. *J Am Soc Mass Spectrom* 2003;14:1290-4.
- Dawson JHJ, Guilhaus M.** Orthogonal-acceleration time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 1989;3:155-9.
- Decaestecker TN, Clauwaert KM, Van Bocxlaer JF, Lambert WE, Van den Eeckhout EG, Van Peteghem CH, De Leenheer AP.** Evaluation of automated single mass spectrometry to tandem mass spectrometry function switching for comprehensive drug profiling analysis using a quadrupole time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 2000;14:1787-92.
- Decaestecker TN, Coopman EM, Van Peteghem CH, Van Bocxlaer JF.** Suitability testing of commercial solid-phase extraction sorbents for sample clean-up in systematic toxicological analysis using liquid chromatography-(tandem) mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci* 2003;789:19-25.
- Decaestecker TN, Vande Casteele SR, Walle-macq PE, Van Peteghem CH, Defore DL, Van Bocxlaer JF.** Information-dependent acquisition-mediated LC-MS/MS screening procedure with semiquantitative potential. *Anal Chem* 2004;76:6365-73.
- Demme U.** Liquid-liquid-extraction in toxicological analysis - old-fashioned or still up-to-date? *Res Legal Med* 2003;30:67-79.
- Deng Y, Wu JT, Zhang H, Olah TV.** Quantitation of drug metabolites in the absence of pure metabolite standards by high-performance liquid chromatography coupled with a chemiluminescence nitrogen detector and

- mass spectrometer. *Rapid Comm Mass Spectrom* 2004;18:1681-5.
- Dolan K, Rouen D, Kimber J.** An overview of the use of urine, hair, sweat and saliva to detect drug use. *Drug Alcohol Rev* 2004;23:213-7.
- Drummer OH.** Chromatographic screening techniques in systematic toxicological analysis. *J Chromatogr B Biomed Sci Appl* 1999;733:27-45.
- Drummer OH.** Requirements for bioanalytical procedures in postmortem toxicology. *Anal Bioanal Chem* 2007;388:1495-503.
- Eckers C, Wolff JC, Haskins NJ, Sage AB, Giles K, Bateman R.** Accurate mass liquid chromatography/mass spectrometry on orthogonal acceleration time-of-flight mass analyzers using switching between separate sample and reference sprays. 1. Proof of concept. *Anal Chem* 2000;72:3683-8.
- Edlund PO, Baranczewski P.** Identification of BVT.2938 metabolites by LC/MS and LC/MS/MS after in vitro incubations with liver microsomes and hepatocytes. *J Pharm Biomed Anal* 2004;34:1079-90.
- Ehrenthal W, Pflieger K, Stuebing G.** The use of exact mass measurement with a GC-MS-instrument in toxicological analysis. *Acta Pharmacol Toxicol* 1977;41 Suppl 2:199-218.
- Elliott SP, Hale KA.** Applications of an HPLC-DAD drug-screening system based on retention indices and UV spectra. *J Anal Toxicol* 1998;22:279-89.
- European union.** Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning performance of analytical methods and the interpretation of results. *Off J Eur Commun* 2002, L221/8.
- Fabbri A, Marchesini G, Morselli-Labate AM, Ruggeri S, Fallani M, Melandri R, Bua V, Pasquale A, Vandelli A.** Comprehensive drug screening in decision making of patients attending the emergency department for suspected drug overdose. *Emerg Med J* 2003;20:25-8.
- Fang AS, Vouros P, Stacey CC, Kruppa GH, Laukien FH, Wintner EA, Carell T, Rebek J.** Rapid characterization of combinatorial libraries using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Comb Chem High Throughput Screen* 1998;1:23-33.
- Fang L, Wan M, Pennacchio M, Pan J.** Evaluation of evaporative light-scattering detector for combinatorial library quantitation by reversed phase HPLC. *J Comb Chem* 2000;2:254-7.
- Fang L, Pan J, Yan B.** High-throughput determination of identity, purity, and quantity of combinatorial library members using LC/MS/UV/ELSD. *Biotechnol Bioeng* 2001;71:162-71.
- Fang L, Cournoyer J, Demee M, Zhao J, Tokushige D, Yan B.** High-throughput liquid chromatography ultraviolet/mass spectrometric analysis of combinatorial libraries using an eight-channel multiplexed electrospray time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 2002;16:1440-7.
- Fang L, Demee M, Cournoyer J, Sierra T, Young C, Yan B.** Parallel high-throughput accurate mass measurement using a nine-channel multiplexed electrospray liquid chromatography ultraviolet time-of-flight mass spectrometry system. *Rapid Commun Mass Spectrom* 2003;17:1425-32.
- Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM.** Electrospray ionization-principles and practice. *Mass Spectrom Rev* 1990;9:37-70.

- Ferrer I, Thurman EM.** Multi-residue method for the analysis of 101 pesticides and their degradation products in food and water samples by liquid chromatography/time-of-flight mass spectrometry. *J Chromatogr A* 2007;1175:24-37.
- Ferrer I, Thurman EM, Zweigenbaum JA.** Screening and confirmation of 100 pesticides in food samples by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2007;21:3869-82.
- Flanagan RJ, Connally G, Evans JM.** Analytical toxicology: Guidelines for sample collection postmortem. *Toxicol Rev* 2005;24:63-71.
- Flanagan RJ, Morgan PE, Spencer EP, Whelp-ton R.** Micro-extraction techniques in analytical toxicology: short review. *Biomed Chromatogr* 2006;20:530-8.
- Ford DL, Kennard W.** Vaporization analyzer. *J Oil Colour Chem Assoc* 1966;49:299-313.
- Fujinari EM, Courthaudon LO.** Nitrogen-specific liquid chromatography detector based on chemiluminescence: application to the analysis of ammonium nitrogen in waste water. *J Chromatogr* 1992;592:209-14.
- Gergov M, Robson JN, Duchoslav E, Ojan-perä I.** Automated liquid chromatographic/tandem mass spectrometric method for screening beta-blocking drugs in urine. *J Mass Spectrom* 2000;35:912-8.
- Gergov M, Boucher B, Ojanperä I, Vuori E.** Toxicological screening of urine for drugs by liquid chromatography/time-of-flight mass spectrometry with automated target library search based on elemental formulas. *Rapid Commun Mass Spectrom* 2001a;15:521-6.
- Gergov M, Robson JN, Ojanperä I, Heinonen OP, Vuori E.** Simultaneous screening and quantitation of 18 antihistamine drugs in blood by liquid chromatography ion spray tandem mass spectrometry. *Forensic Sci Int* 2001b;121:108-15.
- Gergov M, Ojanperä I, Vuori E.** Simultaneous screening for 238 drugs in blood by liquid chromatography-ion spray tandem mass spectrometry with multiple-reaction monitoring. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003;795:41-53.
- Gergov M, Weinmann W, Meriluoto J, Uusitalo J, Ojanperä I.** Comparison of product ion spectra obtained by liquid chromatography/triple-quadrupole mass spectrometry for library search. *Rapid Commun Mass Spectrom* 2004;18:1039-46.
- Goeringer KE, Logan BK, Christian GD.** Identification of tramadol and its metabolites in blood from drug-related deaths and drug-impaired drivers. *J Anal Toxicol* 1997;21:529-37.
- Gorecki T, Lynen F, Szucs R, Sandra P.** Universal response in liquid chromatography using charged aerosol detection. *Anal Chem* 2006;78:3186-92.
- Guilhaus M, Mlynski V, Selby D.** Perfect timing: Time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 1997;11:951-62.
- Guilhaus M, Selby D, Mlynski V.** Orthogonal acceleration time-of-flight mass spectrometry. *Mass Spectrom Rev* 2000;19:65-107.
- Hartstra J, Franke JP, de Zeeuw RA.** How to approach substance identification in qualitative bioanalysis. *J Chromatogr B Biomed Sci Appl* 2000;739:125-37.
- Henderson GL.** Designer drugs: past history and future prospects. *J Forensic Sci* 1988;33:569-75.
- Henry JA.** A fatal toxicity index for antidepressant poisoning. *Acta Psychiatr Scand Suppl* 1989;354:37-45.

- Herzler M, Herre S, Pragst F.** Selectivity of substance identification by HPLC-DAD in toxicological analysis using a UV spectra library of 2682 compounds. *J Anal Toxicol* 2003;27:233-42.
- Hibbert DB.** Scientist vs the law. *Accredit Quality Assur* 2003;8:179-83.
- Hill DW, Kertesz TM, Fontaine D, Friedman R, Grant DF.** Mass spectral metabonomics beyond elemental formula: Chemical database querying by matching experimental with computational fragmentation spectra. *Anal Chem* 2008;80:5574-82.
- Hogenboom AC, van Leerdam JA, de Voogt P.** Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry. *J Chromatogr A* 2008 doi:10.1016/j.chroma.2008.08.053
- Hoja H, Marquet P, Verneuil B, Lotfi H, Péni-caut B, Lachâtre G.** Applications of liquid chromatography-mass spectrometry in analytical toxicology: a review. *J Anal Toxicol* 1997;21:116-26.
- Hopley C, Bristow T, Lubben A, Simpson A, Bull E, Klagkou K, Herniman J, Langley J.** Towards a universal product ion mass spectral library - reproducibility of product ion spectra across eleven different mass spectrometers. *Rapid Commun Mass Spectrom* 2008;22:1779-86.
- Hsu BH, Orton E, Tang SY, Carlton RA.** Application of evaporative light scattering detection to the characterization of combinatorial and parallel synthesis libraries for pharmaceutical drug discovery. *J Chromatogr B Biomed Sci Appl* 1999;725:103-12.
- Hudson P, Barringer M, McBay AJ.** Fatal poisoning with propoxyphene: report from 100 consecutive cases. *South Med J* 1977;70:938-42.
- Huizer H, Poortman-van der Meer AJ, van Egmond HE.** Quantitation of amphetamine-type compounds for which no reference compound is available: the validation of a theoretical model. *Sci Justice* 2001;41:185-92.
- Ibáñez M, Sancho JV, Pozo OJ, Niessen W, Hernández F.** Use of quadrupole time-of-flight mass spectrometry in the elucidation of unknown compounds present in environmental water. *Rapid Commun Mass Spectrom* 2005;19:169-78.
- IST.** *SPE Application note for extraction of acidic, neutral and basic drugs from urine.* International Sorbent Technology, Hengoed, U.K., 1997; IST 1044 A.
- Jones AW, Holmgren A, Kugelberg FC.** Driving under the influence of opiates: concentration relationships between morphine, codeine, 6-acetyl morphine, and ethyl morphine in blood. *J Anal Toxicol* 2008;32:265-72.
- Kavanagh PE.** Program for elemental analysis using low or medium resolution mass-spectra. *Org Mass Spectrom* 1980;15:334-5.
- Kibbey CE.** Quantitation of combinatorial libraries of small organic molecules by normal-phase HPLC with evaporative light-scattering detection. *Mol Divers* 1996;1:247-58.
- Kimble BJ, Cox RE, McPherron RV, Olsen RW, Roitman E, Walls FC, Burlingame AL.** Real-time gas chromatography/high resolution mass spectrometry and its application to the analysis of physiological fluids. *J Chromatogr Sci* 1974;12:647-55.
- Kind T, Fiehn O.** Metabolomic database annotations via query of elemental compositions: mass accuracy is insufficient even at less than 1 ppm. *BMC Bioinformatics* 2006;7:234.
- King LA, Clarke K, Orpet AJ.** Amphetamine profiling in the UK. *Forensic Sci Int* 1994;69:65-75.

- King LA.** Drug content of powders and other illicit preparations in the UK. *Forensic Sci Int* 1997;85:135-47.
- Koski A, Vuori E, Ojanperä I.** Newer antidepressants: evaluation of fatal toxicity index and interaction with alcohol based on Finnish postmortem data. *Int J Legal Med* 2005;119:344-8.
- Kugelberg FC, Jones AW.** Interpreting results of ethanol analysis in postmortem specimens: a review of the literature. *Forensic Sci Int* 2007;165:10-29.
- Kuورانne T, Pystynen KH, Thevis M, Leinonen A, Schanzer W, Kostianen R.** Screening of in vitro synthesised metabolites of 4,9,11-trien-3-one steroids by liquid chromatography-mass spectrometry. *Eur J Mass Spectrom* 2008;14:181-9.
- Kölliker S, Oehme M.** Structure elucidation of nanogram quantities of unknown designer drugs based on phenylalkylamine derivatives by ion trap multiple mass spectrometry. *Anal Bioanal Chem* 2004;378:1294-304.
- Laks S, Pelander A, Vuori E, Pelzing M, Ojanperä I.** Comprehensive urine drug screening by LC/TOFMS based on accurate mass determination and true isotope patterns. *21st Montreux LC/MS Symposium, Abstracts of Poster Presentations*, 2004. p. 102.
- Lane S, Boughtflower B, Mutton I, Paterson C, Farrant D, Taylor N, Blaxill Z, Carmody C, Borman P.** Toward single-calibrant quantification in HPLC. A comparison of three detection strategies: evaporative light scattering, chemiluminescent nitrogen, and proton NMR. *Anal Chem* 2005;77:4354-65.
- Lane S, Boughtflower B, Mutton I, Paterson C, Farrant D, Taylor N, Blaxill Z, Carmody C, Borman P.** Single calibrant quantification with HPLC? Fact or fantasy? *LC-GC Eur* 2006;3:161-70.
- LeBeau MA.** Guidance for improved detection of drugs used to facilitate crimes. *Ther Drug Monit* 2008;30:229-33.
- Leclercq L, Delatour C, Hoes I, Brunelle F, Labrique X, Castro-Perez J.** Use of a five-channel multiplexed electrospray quadrupole time-of-flight hybrid mass spectrometer for metabolite identification. *Rapid Commun Mass Spectrom* 2005;19:1611-8.
- Letot E, Koch G, Falchetto R, Bovermann G, Oberer L, Roth H-J.** Quality control in combinatorial chemistry: Determinations of amounts and comparison of the "purity" of LC-MS-purified samples by NMR, LC-UV and CLND. *J Comb Chem* 2005;7:364-71.
- Lewis S, Kenyon CN, Meili J, Burlingame AL.** High resolution gas chromatographic/real-time high resolution mass spectrometric identification of organic acids in human urine. *Anal Chem* 1979;51:1275-85.
- Liang X, Patel H, Young J, Shah P, Raglione T.** The practical application of implementing the equimolar response principle of chemiluminescent nitrogen detection in pharmaceutical analysis. *J Pharm Biomed Anal* 2008;47:723-30.
- Lillsunde P, Haavanlammi K, Partinen R, Mukkala K, Lamberg M.** Finnish guidelines for workplace drug testing. *Forensic Sci Int* 2008;174:99-102.
- Lucy CA, Harrison CR.** Chemiluminescence nitrogen detection in ion chromatography for the determination of nitrogen-containing anions. *J Chromatogr A* 2001;920:135-41.
- Maier RD, Bogusz M.** Identification power of a standardized HPLC-DAD system for systematic toxicological analysis. *J Anal Toxicol* 1995;19:79-83.
- Maizels M, Budde WL.** Exact mass measurements for confirmation of pesticides and

- herbicides determined by liquid chromatography/time-of-flight mass spectrometry. *Anal Chem* 2001;73:5436-40.
- Makarov A, Denisov E, Kholomeev A, Balchun W, Lange O, Strupat K, Horning S.** Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer. *Anal Chem* 2006;78:2113-20.
- Marquet P, Venisse N, Lacassie E, Lachatre G.** In-source CID mass spectral libraries for the "general unknown" screening of drugs and toxicants. *Analisis* 2000;28:925-34.
- Marquet P, Saint-Marcoux F, Gamble TN, Leblanc JC.** Comparison of a preliminary procedure for the general unknown screening of drugs and toxic compounds using a quadrupole-linear ion-trap mass spectrometer with a liquid chromatography-mass spectrometry reference technique. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003;789:9-18.
- Marshall AG, Hendrikson CL, Jackson GS.** Fourier transform ion cyclotron resonance mass spectrometry: A primer. *Mass Spectrom Rev* 1998;17:1-35.
- Massart DL.** The use of information theory for evaluating the quality of thin-layer chromatographic separations. *J Chromatogr A* 1973;79:157-163.
- Maurer HH.** Position of chromatographic techniques in screening for detection of drugs or poisons in clinical and forensic toxicology and/or doping control. *Clin Chem Lab Med* 2004;42:1310-24.
- Maurer HH.** Current role of liquid chromatography-mass spectrometry in clinical and forensic toxicology. *Anal Bioanal Chem* 2007;388:1315-25.
- May W, Parris R, Beck C, Fassett J, Greenberg R, Guenther F, Kramer G, Wise S, Gills T, Colbert J, Gettings R, MacDonald B.** Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements. NIST Special Publication 260-136. Gaithersburg: National Institute of Standards and Technology; 2000.
- McCarthy R, Gamache P, Asa D.** Compound purity assessment and impurity testing with corona cad. *GIT Lab J Eur* 2005;9:26-7.
- Mirgorodskaya OA, Shevchenko AA, Chernushevich IV, Dodonov AF, Miroshnikov AI.** Electrospray-ionization time-of-flight mass spectrometry in protein chemistry. *Anal Chem* 1994;66:99-107.
- Moffat AC, Owen P, Brown C.** Evaluation of weighted discriminating power calculations as an aid to the selection of chromatographic systems for the analyses of drugs. *J Chromatogr* 1978;161:179-185.
- Moffat AC, Osselton MD, Widdop B,** eds. *Clarke's Analysis of Drugs and Poisons*, 3rd ed. London: Pharmaceutical Press, 2003.
- Morris HR, Paxton T, Dell A, Langhorne J, Berg M, Bordoli RS, Hoyes J, Bateman RH.** High sensitivity collisionally-activated decomposition tandem mass spectrometry on a novel quadrupole/orthogonal-acceleration time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 1996;10:889-96.
- Mueller CA, Weinmann W, Dresen S, Schreiber A, Gergov M.** Development of a multi-target screening analysis for 301 drugs using a QTrap liquid chromatography/tandem mass spectrometry system and automated library searching. *Rapid Commun Mass Spectrom* 2005;19:1332-8.
- Mullett WM.** Determination of drugs in biological fluids by direct injection of samples for liquid-chromatographic analysis. *J Biochem Biophys Methods* 2007;70:263-73.

- Nassar AE, Adams PE.** Metabolite characterization in drug discovery utilizing robotic liquid-handling, quadruple time-of-flight mass spectrometry and in-silico prediction. *Curr Drug Metab* 2003;4:259-71.
- Nielen MW, Vissers JP, Fuchs RE, van Velde JW, Lommen A.** Screening for anabolic steroids and related compounds in illegal cock-tails by liquid chromatography/time-of-flight mass spectrometry and liquid chromatography/quadrupole time-of-flight tandem mass spectrometry with accurate mass measurement. *Rapid Commun Mass Spectrom* 2001;15:1577-85.
- Nielen MW, van Engelen MC, Zuiderent R, Ramaker R.** Screening and confirmation criteria for hormone residue analysis using liquid chromatography accurate mass time-of-flight, Fourier transform ion cyclotron resonance and orbitrap mass spectrometry techniques. *Anal Chim Acta* 2007;586:122-9.
- Nussbaum MA, Baertschi SW, Jansen PJ.** Determination of relative UV response factors for HPLC by use of a chemiluminescent nitrogen-specific detector. *J Pharm Biomed Anal* 2002;27:983-93.
- Ohta H, Suzuki S, Ogasawara K.** Studies on fentanyl and related compounds IV. Chromatographic and spectrometric discrimination of fentanyl and its derivatives. *J Anal Toxicol* 1999;23:280-5.
- Ojanperä I, Pelander A, Laks S, Gergov M, Vuori E, Witt M.** Application of accurate mass measurement to urine drug screening. *J Anal Toxicol* 2005;29:34-40.
- Paar WD, Frankus P, Dengler HJ.** The metabolism of tramadol by human liver microsomes. *Clin Investig* 1992;70:708-10.
- Palmer PT, Enke CG.** Programs for molecular formula determination employing data from unit resolution MS/MS spectra. *Int J Mass Spectrom Ion Process* 1989;88:81-95.
- Paschlau J.** Corona CAD. A new detection method for the HPLC. *GIT Labor-Fachz* 2005;49:32-3.
- Peters FT, Schaefer S, Staack RF, Kraemer T, Maurer HH.** Screening for and validated quantification of amphetamines and of amphetamine- and piperazine-derived designer drugs in human blood plasma by gas chromatography/mass spectrometry. *J Mass Spectrom* 2003;38:659-76.
- Peters FT, Drummer OH, Musshoff F.** Validation of new methods. *Forensic Sci Int* 2007;165:216-24.
- Pihlaine K, Sippola E, Kostianen R.** Rapid identification and quantitation of compounds with forensic interest using fast liquid chromatography-ion trap mass spectrometry and library searching. *J Chromatogr A* 2003;994:93-102.
- Polettini A, Gottardo R, Pascali JP, Tagliaro F.** Implementation and performance evaluation of a database of chemical formulas for the screening of pharmaco/toxicologically relevant compounds in biological samples using electrospray ionization-time-of-flight mass spectrometry. *Anal Chem* 2008;80:3050-7.
- Poortman-van der Meer AJ, Huizer H.** A contribution to the improvement of accuracy in the quantitation of THC. *Forensic Sci Int* 1999;101:1-8.
- Pragst F, Herzler M, Erxleben BT.** Systematic toxicological analysis by high-performance liquid chromatography with diode array detection (HPLC-DAD). *Clin Chem Lab Med* 2004;42:1325-40.
- Pragst F.** Application of solid-phase microextraction in analytical toxicology. *Anal Bioanal Chem* 2007;388:1393-414.

- Rasanen I, Kontinen I, Nokua J, Ojanpera I, Vuori E.** Precise gas chromatography with retention time locking in comprehensive toxicological screening for drugs in blood. *J Chromatogr B Anal Technol Biomed Life Sci* 2003;788:243-50.
- Reiter A, Hake J, Meissner C, Rohwer J, Friedrich HJ, Oehmichen M.** Time of drug elimination in chronic drug abusers. Case study of 52 patients in a "low-step" detoxification ward. *Forensic Sci Int* 2001;119:248-53.
- Rittner M, Pragst F, Bork WR, Neumann J.** Screening method for seventy psychoactive drugs or drug metabolites in serum based on high-performance liquid chromatography--electrospray ionization mass spectrometry. *J Anal Toxicol* 2001;25:115-24.
- Rivier L.** Criteria for the identification of compounds by liquid chromatography--mass spectrometry and liquid chromatography--multiple mass spectrometry in forensic toxicology and doping analysis. *Anal Chim Acta* 2003;492:69-82.
- Robbat A, Corso NP, Liu TY.** Evaluation of a nitrosyl-specific gas-phase chemiluminescence detector with high-performance liquid chromatography. *Anal Chem* 1988;60:173-4.
- Rockwood AL, van Orden SL.** Ultrahigh-speed calculation of isotope distributions. *Anal Chem* 1996;68:2027-30.
- Rockwood AL, Kushnir MM, Nelson GJ.** Dissociation of individual isotopic peaks: predicting isotopic distributions of products in MSn. *J Am Soc Mass Spectrom* 2003;14:311-22.
- Rouini MR, Ardakani YH, Soltani F, Aboul-Enein HY, Foroumadi A.** Development and validation of a rapid HPLC method for simultaneous determination of tramadol, and its two main metabolites in human plasma. *J Chromatogr B* 2006;830:207-211.
- Saint-Marcoux F, Lachâtre G, Marquet P.** Evaluation of an improved general unknown screening procedure using liquid chromatography-electrospray-mass spectrometry by comparison with gas chromatography and high-performance liquid-chromatography-diode array detection. *J Am Soc Mass Spectrom* 2003;14:14-22.
- Saint-Marcoux F, Sauvage FL, Marquet P.** Current role of LC-MS in therapeutic drug monitoring. *Anal Bioanal Chem* 2007;388:1327-49.
- Schulz M, Schmoldt A.** Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. *Pharmazie* 2003;58:447-74.
- Shah N, Gao M, Tsutsui K, Lu A, Davis J, Scheuerman R, Fitch WL, Wilgus RL.** A novel approach to high-throughput quality control of parallel synthesis libraries. *J Comb Chem* 2000a;2:453-6.
- Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ, McKay G, Miller KJ, Patnaik RN, Powell ML, Tonelli A, Viswanathan CT, Yacobi A.** Bioanalytical method validation--a revisit with a decade of progress. *Pharm Res* 2000b;17:1551-7.
- Shulgin AT.** In *Hallucinogens. A Forensic Handbook*, Laing R., ed. Amsterdam: Academic Press, 2003. pp 67-137.
- Siek TJ.** Effective use of organic solvents to remove drugs from biologic specimens. *Clin Toxicol* 1978;13:205-30.
- Slobodník J, Hogenboom AC, Vreuls JJ, Rontree JA, van Baar BLM, Niessen WMA, Brinkman UAT.** Trace-level determination of pesticide residues using on-line solid-phase extraction-column liquid chromatography with atmospheric pressure ionization mass spectrometric and tandem mass spectrometric detection. *J Chromatogr A* 1996;741:59-74.

- Smith RD, Anderson GA, Lipton MS, Pasa-Tolic L, Shen Y, Conrads TP, Veenstra TD, Udseth HR.** An accurate mass tag strategy for quantitative and high-throughput proteome measurements. *Proteomics* 2002;2:513-23.
- Smith ML, Vorce SP, Holler JM, Shimomura E, Magluilo J, Jacobs AJ, Huestis MA.** Modern instrumental methods in forensic toxicology. *J Anal Toxicol* 2007;31:237-53.
- Springer D, Fritschi G, Maurer HH.** Metabolism of the new designer drug alpha-pyrrolidinopropiophenone (PPP) and the toxicological detection of PPP and 4'-methyl-alpha-pyrrolidinopropiophenone (MPPP) studied in rat urine using gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003;796:253-66.
- Stead AH, Moffat AC.** Quantification of the interaction between barbiturates and alcohol and interpretation of fatal blood concentrations. *Hum Toxicol* 1983;2:5-14.
- Stranz DD, Miao S, Campbell S, Maydwell G, Ekins S.** Combined computational metabolite prediction and automated structure-based analysis of mass spectrometric data. *Toxicol Mech Methods* 2008;18:243-50.
- Stroh JG, Petucci CJ, Brecker SJ, Huang N, Lau JM.** Automated sub-ppm mass accuracy on an ESI-TOF for use with drug discovery compound libraries. *J Am Soc Mass Spectrom* 2007;18:1612-6.
- Sweeney DL.** A systematic computational approach for identifying small molecules from accurate-mass fragmentation data. *Am Lab* 2007;39:12-4.
- Taylor EW, Qian MG, Dollinger GD.** Simultaneous online characterization of small organic molecules derived from combinatorial libraries for identity, quantity, and purity by reversed-phase HPLC with chemiluminescent nitrogen, UV, and mass spectrometric detection. *Anal Chem* 1998;70:3339-47.
- Taylor EW, Jia W, Bush M, Dollinger GD.** Accelerating the drug optimization process: identification, structure elucidation, and quantification of in vivo metabolites using stable isotopes with LC/MS and the chemiluminescent nitrogen detector. *Anal Chem* 2002;74:3232-8.
- Tenhosaari A.** Computer-assisted composition analysis of unknown compounds by simultaneous analysis of the intensity ratios of isotope patterns of the molecular ion and daughter ions in low-resolution mass spectrometry. *Org Mass Spectrom* 1988;23:236-9.
- Thevis M, Opfermann G, Schänzer W.** High speed determination of beta-receptor blocking agents in human urine by liquid chromatography/tandem mass spectrometry. *Biomed Chromatogr* 2001;15:393-402.
- Thevis M, Kohler M, Schänzer W.** New drugs and methods of doping and manipulation. *Drug Discov Today* 2008;13:59-66.
- Thurman EM, Ferrer I, Fernández-Alba AR.** Matching unknown empirical formulas to chemical structure using LC/MS TOF accurate mass and database searching: example of unknown pesticides on tomato skins. *J Chromatogr A* 2005;1067:127-34.
- Ting KC, Zhou EG, Saini N.** Determination of imidacloprid in fruits and vegetables by liquid chromatography with diode array and nitrogen-specific chemiluminescence detection. *J AOAC Int* 2004;87:997-1002.
- Tjäderborn M, Jönsson AK, Hägg S, Ahlner J.** Fatal unintentional intoxications with tramadol during 1995-2005. *Forensic Sci Int* 2007;173:107-11.
- Tracqui A, Kintz P, Ludes B.** Buprenorphine-related deaths among drug addicts in France: a report on 20 fatalities. *J Anal Toxicol* 1998;22:430-4.

- Tuomisto J, Pekkanen J, Kiviranta H, Tukiainen E, Vartiainen T, Viluksela M, Tuomisto JT.** Dioxin Cancer Risk - Example of Hormesis? *Dose Response* 2006;3: 332-41.
- Tyler AN, Clayton E, Green BN.** Exact mass measurement of polar organic molecules at low resolution using electrospray ionization and a quadrupole mass spectrometer. *Anal Chem* 1996;68:3561-9.
- Van Bocxlaer JF, Clauwaert KM, Lambert WE, Deforce DL, Van den Eeckhout EG, De Leenheer AP.** Liquid chromatography-mass spectrometry in forensic toxicology. *Mass Spectrom Rev* 2000;19:165-214.
- Van Bocxlaer JF.** Recent trends in analytical procedures in forensic toxicology. *Ther Drug Monit* 2005;27:752-5.
- Verstraete AG.** Detection times of drugs of abuse in blood, urine, and oral fluid. *Ther Drug Monit* 2004;26:200-5.
- Vistoli G, Pedretti A, Testa B.** Assessing drug-likeness - what are we missing? *Drug Discov Today* 2008;13:285-94.
- Walsh JM, de Gier JJ, Christopherson AS, Verstraete AG.** Drugs and driving. *Traffic Inj Prev* 2004;5:241-53.
- Webb K, Bristow T, Sargent M, Stein B,** eds. *Methodology for accurate mass measurement of small molecules. Best practice guide.* Teddington: LGC Limited, 2004. URL www.bmss.org.uk/Docs/VIMMS_guide.pdf (accessed August 2008)
- Weinmann W, Wiedemann A, Eppinger B, Renz M, Svoboda M.** Screening for drugs in serum by electrospray ionization/collision-induced dissociation and library searching. *J Am Soc Mass Spectrom* 1999;10:1028-37.
- Weinmann W, Gergov M, Goerner M.** MS/MS-libraries with triple quadrupole-tandem mass spectrometers for drug identification and drug screening. *Analysis* 2000;28:934-41.
- Wennig R.** Threshold values in toxicology - useful or not? *Forensic Sci Int* 2000;113:323-30.
- Weyermann C, Marquis R, Delaporte C, Esseiva P, Lock E, Aalberg L, Bozenko JS Jr, Dieckmann S, Dujourdy L, Zrcek F.** Drug intelligence based on MDMA tablets data I. Organic impurities profiling. *Forensic Sci Int* 2008;177:11-6.
- Wiley WC, McClaren IH.** Time-of-flight mass spectrometer with improved resolution. *Rev Sci Instrum* 1955;26:1150-7.
- Wille SM, Lambert WE.** Recent developments in extraction procedures relevant to analytical toxicology. *Anal Bioanal Chem* 2007;388:1381-91.
- Yan X.** Detection by ozone-induced chemiluminescence in chromatography. *J Chromatogr A* 1999;842:267-308.
- Yan B, Fang L, Irving M, Zhang S, Boldi AM, Woolard F, Johnson CR, Kshirsagar T, Figliozzi GM, Krueger CA, Collins N.** Quality control in combinatorial chemistry: Determination of the quantity, purity, and quantitative purity of compounds in combinatorial libraries. *J Comb Chem* 2003;5:547-59.
- Yan B, Zhao J, Leopold K, Zhang B, Jiang G.** Structure-dependent response of a chemiluminescence nitrogen detector for organic compounds with adjacent nitrogen atoms connected by a single bond. *Anal Chem* 2007;79:718 -26.
- Yurek DA, Branch DL, Kuo MS.** Development of a system to evaluate compound identity, purity, and concentration in a single experiment and its application in quality assessment of combinatorial libraries and screening hits. *J Comb Chem* 2002;4:138-48.

de Zeeuw RA. Substance identification: the weak link in analytical toxicology. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;811:3-12.

Zhang H, Heinig K, Henion J. Atmospheric pressure ionization time-of-flight mass spectrometry coupled with fast liquid chromatography for quantitation and accurate mass measurement of five pharmaceutical drugs in human plasma. *J Mass Spectrom* 2000a;35:423-31.

Zhang H, Henion J, Yang Y, Spooner N. Application of atmospheric pressure ionization time-of-flight mass spectrometry coupled with liquid chromatography for the characterization of in vitro drug metabolites. *Anal Chem* 2000b;72:3342-8.

Zhang B, Li X, Yan B. Advances in HPLC detection--towards universal detection. *Anal Bioanal Chem* 2008;390:299-301.